Characterization of bacterial antagonists of *Rhizoctonia solani* and *Fusarium oxysporum* from six European soils and their potential application for biological control

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Summary

Due to the harmful effect of chemical pesticides on the environment and human health, the use of microorganisms with natural activity against plant pathogens in plant protection has been considered as an appealing and ecological friendly alternative. As a result, microorganisms with natural activity against plant pathogens have been isolated in different environments, for example in normal agricultural soils and particularly in suppressive soils, where their population and activity have been reported to be very significant in natural control of plant pathogens and diseases.

The major aim of the thesis work was to characterize the antagonistic potential of culturable bacteria in six agricultural soils from different geographic locations in Europe (France, FR; the Netherlands, NL; Sweden, SE; the United Kingdom, UK; and two sites in Germany, Berlin, G-BR and Braunschweig, G-BS). Four of the soils (FR, NL, SE, UK) have previous documentation of disease suppression while two (G-BR and G-BS) have no record of diseases suppression.

A total of 1,788 bacterial isolates were obtained on three different media (R2A, KMB, and AGS) from all the six soils (approximately 100 isolates per medium and site) and were screened *in vitro* in a dual culture assay for antagonistic activity towards two phytopathogenic fungi {*Rhizoctonia solani* AG3 and *Fusarium oxysporum* f. sp. *lini* (Foln3)}; in all 327 antagonistic isolates were found. The overall proportion of antagonists was higher in three of the suppressive soils (FR, NL and SE) with NL having the highest proportion. Phenotypic characterization of the antagonists according to chitinase, glucanase, cellulase, protease and siderophores production revealed siderophores and protease activity as the most prominent traits among the antagonists. Even though, FAME and 16S rRNA gene sequencing identified majority

of the antagonists as *Pseudomonas* spp. (110 of 327) and *Streptomyces* spp. (113 of 327), phenotypic and genotypic diversity among the antagonists were found.

To further elucidate the probable mechanisms of activity of the antagonists, the second largest bacterial group found in our collection of antagonists, the *Pseudomonas spp.* were screened by PCR-Southern blot hybridization for the presence of genes involved in biosynthesis of antibiotics, which activity in biological control have been reported, 2,4-diacetylphloroglucinol (*phlD*), pyrrolnitrin (*prnD*), pyoluteorin (*pltC*) and phenazine (*phzCD*). Although *Pseudomonas* antagonists carrying *phlD* gene were found in all sites, by far the highest proportion was detected in SE, where about 50% of the *Pseudomonas* antagonists contained this gene. *Pseudomonas* antagonists containing the *prnD* gene were only represented scarcely in four of the soil, while *phzCD or pltC* genes were not found. In addition, grouping of the antagonists according to the PCR-DGGE mobility of the *Pseudomonas*-specific global regulatory antibiotics and cyanide (*gacA)* gene showed that the gene was highly diverse among the collection of *Pseudomonas* antagonists obtained from each site. Although a higher genotypic diversity among the *Pseudomonas* antagonists was found with BOX-PCR than with gacA-DGGE grouping, the resolution of gacA-DGGE method is by far better than 16S RNA gene based analysis. When *gacA* types derived from the culturable *Pseudomonas* antagonists were linked with the culture-independent *gacA*-DGGE profiles from community DNA of each soil, only few representatives of the culture-derived *gacA* types in their corresponding *gacA* community patterns could be matched.

Ten of the antagonists were selected based on their strong or dual *in vitro* antagonistic activity towards *R. solani* and/or *F. oxysporum*, and their root colonization efficiency. Their ability to control bottom rot disease caused by *R. solani*

AG1-IB on lettuce plants was determined in growth chamber experiments. Four of the ten antagonists (three identified as *Pseudomonas fluorescens* and one as *Pseudomonas jessenii*) significantly decreased disease severity in two experiments, of which only *Pseudomonas jessenii* strain RU47 showed effective and consistent pathogen suppression in all the four experiments conducted. Plate counts and DGGE of PCR amplified *Pseudomonas*-specific *gacA* gene fragments from total community DNA of inoculated plants confirmed the ability of RU47 to establish as a dominant *Pseudomonas* population in the rhizosphere of lettuce. Inoculation of RU47 had no effects on the *Pseudomonas* communities, whereas it had a considerable decrease in the relative abundance of the pathogen in the rhizosphere of lettuce.

The present work showed that the bacterial antagonists retrieved from the different soils were phenotypically and genotypically diverse and that the composition found in each soil was site-specific. None of the mechanism of antagonistic activity (hydrolytic enzymes, siderophores production and detection of antibiotic producing genes) investigated in this study could be regarded as specific to a given site, except for a remarkably higher frequency of *phlD* gene detection among *Pseudomonas* antagonists from SE; suggesting they may likely play a significant role in the natural disease suppression of this soil. Furthermore, our results suggest that *P. jessenii* RU47, the most effective and consistent antagonist obtained from the growth chamber experiments, can be considered as a promising biological control agent to suppress *R. solani* AG1-IB in lettuce.

CHAPTER 1

CHAPTER 1

INTRODUCTION

Conventional agriculture depends largely on the use of chemical inputs, such as pesticides and fertilizers, to control plant pathogens and to enhance crop yield. Health concerns and environmental hazards associated with the use of chemical pesticides have resulted in an increasing interest in biological control as a promising alternative or a supplemental way of reducing the use of agro-chemicals. Some naturally occurring soil bacteria and fungi have demonstrated great potential to antagonize crop pathogens, hence, biological control involving the use of such plant-beneficial microorganisms for plant protection is being considered as a viable substitute to reduce the use of chemical pesticides (Compant et al., 2005). In certain soils, such indigenous plant-beneficial microorganisms are able to suppress the growth of certain phytopathogens/parasites without the use of chemical pesticides, and these soils are referred to as `disease suppressive soils` (Weller et al., 2002). Thus, suppressive soils are regarded as sources of antagonistic microorganisms. These plant beneficial microorganisms are known to antagonize phytopathogens through competition for niches or nutrients (e.g. iron through siderophores synthesis); parasitism that may involve production of hydrolytic enzymes, for example, chitinase, glucanase, protease and cellulase that can lyse pathogen cell walls; inhibition of the pathogens by anti-microbial compounds (antibiosis); induction of systemic resistance in host plants (Whipps 2001; Compant et al., 2005). Genes involved in the biosynthesis of many of these secondary metabolites are positively controlled by the GacS/GacA two-component system in several beneficial gram-negative bacteria, particularly, in *Pseudomonas* spp. (Heeb and Haas, 2001). Over the past three

decades studies on the use of beneficial microorganisms as biocontrol agents for plant protection have increased greatly. Several strains have been reported to show good performance *in vitro* and in specific trials, nonetheless, only few have demonstrated consistent and effective biocontrol in different field situations (Kiely et al., 2006). As a result only very few get to the market. Fravel (2005) estimated the number of biocontrol products in the market as 1% of agricultural chemical sales. However, there is need to search for more reliable and consistent biocontrol agents to meet up the increasing demand for chemical residue-free agricultural products. The chance of selecting effective biocontrol agents may be improved by isolating biocontrol strains from the same environment in which they are to be used. Hence, two widely used approaches to select for potential biocontrol agents focus first on isolation of antagonists from soils that are naturally suppressive to a particular pathogen (suppressive soils); the second approach comprises isolation from intended environment of use, such as soils, seeds or roots. In the light of these, the general idea of the study presented in this thesis was to characterize bacteria with antagonistic potential against *R. solani* and *F. oxysporum* from six agricultural soils of European origin. In addition, the taxonomic composition and diversity of the antagonists in each soil were evaluated and the efficacy of some selected antagonists in controlling bottom rot disease of *R. solani* on lettuce plants in growth chamber experiments was assessed.

The results of the study presented in this thesis are described in Chapters 3 – 5. While Chapter 3 has been accepted for publication, Chapter 4 and 5 are to be submitted to a peer-reviewed journal for publication; thus the chapters are written in a manuscript style, each having its own abstract, introduction, materials and methods, results and discussion. Prior to the manuscripts, a background review of literature

was provided, followed by the study outline and aims (Chapter 2). In Chapter 6, general discussion and conclusions of the whole study was presented, followed by a list of references cited in all the Chapters.

CHAPTER 2

CHAPTER 2

BACKGROUND

1. Biological control of plant diseases

Biological control is a strategy that was proposed half a century ago, as a result of several negative effects that the increasing use of agro-chemicals had on the environment, farmers (applicators of the chemicals) and the consumers. These negative effects include a decrease in biodiversity of the soil-inhabiting microorganisms; hazardous effects of pesticides runoff on the aquatic systems (Johnston, 1986); the non-target environmental impacts and the development of resistance to fungicides by pathogens (de Weger et al., 1995; Gerhardson, 2002); acute health problems resulting from exposure of farmers to chemical pesticides (Arcury et al., 2003); pesticides residues in many food crops including fruits and vegetables which endanger the health of the consumers; furthermore, the increasing cost of pesticides, particularly in low-income countries of the world (Gerhardson, 2002). These health and environmental hazards associated with the use of chemical pesticides are of primary concern to international organizations, many governments, employers and workers. Therefore, many countries have banned or severely restricted the use of some very hazardous chemicals, including some pesticides that are used to suppress plant diseases. For instance, Methylbromide (MeBr), an odorless, colorless gas used as agricultural soil and structural fumigant to control a wide variety of pests throughout the world, has been reported by the U.S. Environmental Protection Agency (EPA) to deplete the stratospheric ozone layer and is classified as a class I ozone-depleting substance (EPA, 2006).

Biological control has been described as the reduction of the amount of pathogen inoculum or disease-producing activity of a pathogen accomplished by or

through one or more organisms other than man (Cook and Baker, 1983). According to this definition, organisms and procedures involved in biological control include: (1) avirulent or hypo-virulent individual or population within the pathogenic species, (2) antagonistic microorganisms, and (3) effective resistance of the pathogen by the host plant through manipulation. Plant pathogens and insect pests, among others, are the populations within the pathogenic species that are targeted by biological control. In this dissertation, only three plant pathogens are considered, namely, *Rhizoctonia solani, Fusarium oxysporum* and *Ralstonia solanacearum*. Biological control can be achieved through the following approaches, which aimed at maintaining microbial balance: introduction of antagonists, plant breeding and specific cultural practices (Alabouvette et al., 2006). This dissertation focuses on microbial antagonists as biological control agents. Microbial antagonists include naturally occurring microorganisms that are antagonistic to crop pathogens, and have the potential to protect crop against the harmful effect of the pathogen, consequently providing an alternative to chemical fungicides (Weller et al., 2002; Welbaum et al., 2004; Mark et al., 2006). Both to the environments and to the consumers of agricultural products, the use of microbial antagonists as biological control agents is believed to be safer than the traditional chemical pesticides.

Representatives of a range of bacteria and fungi, and in a few cases nematodes have been identified as biocontrol agents (BCAs) against soil-borne plant pathogens; the most abundant soil and plant-associated bacterial genera among such groups are *Burkholderia, Bacillus, Pseudomonas, Serratia* and *Streptomyces (*Adb-Allah, 2001*;* Berg et al., 2002; Nair et al., 2002; Costa et al., 2006a; Mark et al., 2006*).* In this study, interest is on isolating soil inhabiting bacteria, particularly the

bacterial genera *Pseudomonas* and *Streptomyces,* which have antagonistic potential against soil-borne plant pathogens.

2. *Pseudomonas* species

The genus *Pseudomonas* comprises the relatively large and important group of gram-negative, non-spore forming, motile rod bacteria (Bergey´s Manual of Systemic Bacteriology, second ed. Vol. 2). They are ubiquitous in nature and are one of the best-studied soil-borne bacterial groups. Some members of the genus are characterized by production of diffusible and/or insoluble pigments. Pseudomonads are well-known for their ability to degrade compounds, which are difficult to utilize by other organisms (Khan and Ahmad, 2006). Consequently, they are important organisms in bioremediation. They produce wide varieties of antibiotics, which confer a competitive advantage and microbial fitness to survive in most environments (Haas and Keel, 2003; Paulsen et al., 2005). This genus also comprises human, animal and plant pathogens; besides, there are also important beneficial bacteria such as plant growth promoters and biocontrol agents (Raaijmakers et al., 2002). Due to their ability to produce variable metabolites and to utilize several organic compounds most biocontrol pseudomonads are not specific for one pathogen or plant species only, but have a wide host range and suppress several pathogens. For instance, Siddiqui and Shaukat (2003) reported the suppression of four root-infecting fungi, *Macrophomina phaseolina, Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani* by the biocontrol strain, *Pseudomonas aeruginosa* IE-6 both under laboratory and field conditions. Antagonistic *Pseudomonas* species have been isolated from agricultural soils as well as soils that were naturally suppressive to different plant pathogens, including *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum*, *Rhizoctonia*

solani (de Souza et al., 2003a; Garbeva et al., 2004; Bergsma-Vlami et al., 2005a). Natural biological suppression to take-all disease caused by the fungus *Gaeumannomyces graminis* var*. tritici* in fields cultivated to wheat was associated with the dominance of indigenous populations of root-colonizing fluorescent pseudomonads producing the antimicrobial metabolite 2,4-diacetylphloroglucinol (Raaijmakers and Weller, 1998; de Souza et al, 2003a). Similar observations were made in a Swiss soil suppressive to *Thielaviopsis basicola* (Ramette et al., 2003).

In addition, *Pseudomonas* spp. are common rhizosphere organisms and have been shown to be excellent root colonizers (Lugtenberg et al., 2001; Raaijmakers and Weller, 2001).

3. *Streptomyces* species

The suppressive nature of the bacterial genus, *Streptomyces*, against diverse fungal and bacterial phytopathogens has also been reported in several studies (Gyenis et al., 2003; Michaud et al., 2002; Abd-Allah, 2001; Liu et al., 1996). *Streptomyces* are members of the bacterial class actinomycetes. They resemble fungi in their branching filamentous structure. However, they are true gram-positive bacteria and are characterized by high guanine-plus-cytosine (G + C) content in their genomes. They are non-motile, most of them produce spores and are found predominantly in soil and in decaying vegetation. They persist as saprophytes and are often associated with plant roots. Few species are pathogenic to animals and plants. *Streptomyces* are noted for their distinct “earthy” odor, which results from production of volatile metabolites such as geosmin (Gerber and Lechevalier, 1965). They are well-known producers of antibiotics and extracellular hydrolytic enzymes. In particular, approximately 60% of antibiotics developed for agricultural use were

isolated from *Streptomyces* spp. (Sahin, 2005). Many *Streptomyces* can produce more than one antibiotic and also possess resistance to multiple antibiotics (Davelos et al., 2004). Antibiotics produced by *Streptomyces* have the capability to inhibit diverse soil-borne microbes. In Minnesota, soil under long-time monoculture of potato was found to develop natural suppression to potato scab disease caused by the soil-borne bacterium *Streptomyces scabies* as a result of increased population of antibiotic-producing, non-pathogenic *Streptomyces* in this soil (Weller et al., 2002). Recent reports by Watve et al. (2001) have also shown that this group of microorganisms still remains an important source of antibiotics. These characteristics, as well as their ability to withstand desiccation and high temperatures as spores, make them attractive as biological control agents.

4. Biological control of soil-borne pathogens: the mechanisms involved

Several reports on disease suppression have pointed out that many different mechanisms contribute to disease control. Therefore, it is important to understand the mechanisms of disease suppression by biocontrol agents for the successful utilization of biological control as disease management strategy. In the following some of the recognized mechanisms of biocontrol of soil-borne pathogens by anti-microbial agents will be discussed.

4.1. Competition

Competition for resources such as nutrients, oxygen and colonization site occurs generally in soil between soil-inhabiting organisms. For biocontrol purpose, it occurs when the antagonist directly competes with pathogens for these resources.

Root inhabiting microorganisms compete for infection sites at the root surfaces (Alabouvette, 2006). Competition for nutrients, especially for carbon, is assumed to be responsible for the well-known phenomenon of fungistasis characterizing the inhibition of fungal spore germination in soil (Alabouvette et al., 2006). Couteaudier and Alabouvette (1990) showed clearly that competition for carbon was one of the mechanisms used by the non-pathogenic *F. oxysporum* to suppress pathogenic *F. oxysporum,* the causal agent of fusarium wilt. Competition for trace elements, such as iron, copper, zinc, manganese etc., also occurs in soils. For example, iron is an essential growth element for all living organisms and the scarcity of its bio-available form in soil habitats results in a furious competition (Loper and Henkels**,** 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (also by most biocontrol agents) to solubilize and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow for lack of it (Loper and Henkels**,** 1997; Haas and Defago, 2005). Examples of siderophores produced by biocontrol agents are pyoverdin, salicylic acid and pyochelin (Haas and Defago, 2005). Pyoverdin (also called Pseudobactin), an extracellular diffusible pigment, produced by fluorescent *Pseudomonas* spp. is responsible for their fluorescence (Haas and Defago, 2005). Pyoverdin functions by binding to Fe^{3+} ion (the insoluble and unavailable form of iron in the soil), which is subsequently transported into the cytoplasm of the producing organisms through interacting with a specific outer-membrane receptor. After transportation into the cytoplasm, Fe^{3+} is converted to Fe^{2+} (Haas and Defago, 2005). Siderophore production favors rapid growth of the producing organisms. De Boer et al. (2003) found that the role of siderophores was associated with the antagonistic properties of *Pseudomonas putida* WCS358 in

suppressing fusarium wilt of radish. Some siderophores are also good chelators of some elements other than iron. For example, pyochelin is a good Cu^{2+} and Zn^{2+} chelator (Haas and Defago, 2005). Consequently, siderophores may directly stimulate the production of other anti-microbial compounds, when these elements are increasingly made available to the bacteria (Duffy and Defago, 1999). Under certain conditions, siderophores can function as a diffusible bacteriostatic or fungistatic antibiotic (Haas and Defago, 2005). Even though, various bacterial siderophores differ in their abilities to sequester iron, generally they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity. Kloepper et al. (1980) clearly demonstrated the inhibitory potential of pyoverdin (Pvd)-producing *Pseudomonas* spp. towards bacteria and fungi with less potent siderophores in an iron-depleted medium. Although several authors have demonstrated the contribution of siderophores to disease suppression in some situations; it is believed that siderophores alone are not sufficient to account for suppression; if they were, it would be difficult to explain why most strains which produce siderophores, do not have biocontrol activity (Haas and Defago, 2005).

4.2. Parasitism

Mycoparasitism is a process initiated by physical destruction of the fungal cell wall mediated by the action of hydrolytic enzymes produced by a biocontrol agent (Adams, 1990). Chitin and ß-1,3-glucan are the two major structural components of most plant pathogenic fungi. Antagonists invade pathogens by excretion of extracellular enzymes that can lyse pathogen cell walls or cause degradation of chlamydospores, oospores, conidia, sporangia, and zoospores. Such extracellular enzymes include chitinases, cellulases, proteases and ß-1,3-glucanases. Dunne et

al. (2000) showed that overproduction of extracellular protease in the mutant strains of *Stenotrophomonas maltophilia* W81 resulted in improved biocontrol of *Pythium ultimum.* Excretion of chitinases and glucanases by species of *Trichoderma* and *Streptomyces* has also been shown to play an important role in mycoparasitism of phytopathogenic fungi (Baek et al., 1999; Gomes et al., 2000; Whipps, 2001).

4.3. Induction of plant resistance mechanisms

Expression of natural defense reaction against stresses from biotic or abiotic origin is exhibited by all plants, such as (i) physical stresses (heat or frost), (ii) inoculation by pathogenic or non-pathogenic organisms, (iii) chemical molecules from natural or synthetic origins (Alabouvette, 2006). Early recognition of the aggressor by the plant is one of the mechanisms involved in elicitation of plant defense reactions (Lugtenberg et al., 2002). Recognition of the aggressor immediately initiates a cascade of molecular signals and the transcription of many genes, which eventually results in the production of defence molecules by the plant (van Loon, 2000). Such defence molecules include phytoalexins, pathogenesis-related (PR) proteins (such as chitinases, ß-1,3-glucanases, proteinase inhibitors etc.) and reinforcement of cell walls (van Loon, 2000; Whipps, 2001). Cell wall thickenings, wall appositions or rapid death of the injured plant cells resulting in necrosis of the immediate adjacent tissues are barriers which cut the pathogen off its nutrients and contribute to slowing down of the fungus progressive invasion (Lugtenberg et al., 2002; Alabouvette, 2006). A virulent pathogen inhibits resistance reactions, or circumvents the effects of active defenses. As a result of these natural defense mechanisms, plants are able to produce an immune response after a primary pathogen infection known as systemic acquired resistance (SAR). The host plant can also benefit directly from non-

pathogenic rhizobacteria and fungi through the production of metabolites that either stimulate root development and plant growth or trigger the induction of systemic resistance (ISR) that is phenotypically similar to SAR (van Loon et al. 1998; Bakker et al., 2003). In other words, SAR is a pathogen-induce type of resistance which requires accumulation of salicylic acid while ISR is a rhizobacteria-induced type that depends on responses to ethylene and jasmonic acid (Bakker et al., 2003).

A variety of soil and rhizosphere bacteria and fungal isolates can provide protection against viral, fungal, and bacterial plant pathogens by turning on ISR in plants (van Loon et al., 1998; Whipps, 2001). However, rhizobacteria differ in their ability to turn on ISR, some are active on particular plants and not on the other (Whipps, 2001). These plant defense-inducing bacteria are also known to enhance plant growth and are referred to as plant growth promoting rhizobacteria (PGPR). Siddiqui and Shaukat (2002) showed three PGPR strains, *P. fluorescens* CHAO, *P. aeruginosa* IE-6 S+ and *Bradyrhizobium japonicum* 569Sm(r) not only suppressed root-infecting fungi and root-knot nematodes but also enhanced growth of tomato plants both under glasshouse and field conditions. Even though the full range of metabolites involved in microbially mediated ISR is not yet known, siderophores, antibiotics, and lipopolysaccharides has been clearly indicated (Whipps, 2001; Bakker et al., 2003).

4.4. Antibiosis

It refers to the inhibition or destruction of the pathogen by the metabolic products produced during growth of the antagonist. These products include volatile compounds, toxic compounds and antibiotics, which are deleterious to the growth or metabolic activities of other microorganisms at low concentrations (Fravel, 1988). Several authors have reported on the involvement of antibiosis in biocontrol of plant

pathogens. Mechanism of antagonistic activity by the biocontrol agents, *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f. sp*. cubense* race (Getha and Vikineswary, 2002) and *Pantoea agglomerans* strain Eh252 on *Erwinia amylovora* (causal agent of fire blight in orchards) (Stockwell et al., 2002) was attributed to antibiosis. *In vitro* and *in vivo* production of antibiotics by numerous antagonistic bacterial strains have been demonstrated *(*Bergsma-Vlami et al*.,* 2005a). Raaijmaker et al. (2002) and Chin-A-Woeng et al. (2001) provided a comprehensive list of antibiotics that have been implicated in biocontrol, their producing organisms and the affected pathogens. Among them were 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT) and different derivatives of phenazine (Phz). In this thesis we focus on the genes involved in biosynthesis of these four antibiotics, which production and relevance in biocontrol activity of *Pseudomonas spp*. have been demonstrated in several studies (Chin-A-Woeng et al., 2001; Delaney et al., 2001; Raaijmaker et al., 2002; de Souza et al., 2003a; Ramette et al., 2003).

4.4.1. 2,4-diacetylphloroglucinol

2,4-diacetylphloroglucinol (2,4-DAPG) is a polyketide compound (Fig. 1.1), which has received particular attention because of its broad-spectrum antiviral, antifungal, antibacterial, and antitumor activity and phytotoxic properties (Raaijmaker et al., 2002; Haas and Keel, 2003; Isnansetyo et al., 2003). Even in medical area, there has been increasing interest on the use of 2,4-DAPG, due to its recently reported bacteriolytic activity against multidrug-resistant *Staphylococcus aureus* (Isnansetyo et al., 2003). 2,4-DAPG is synthesized by several plant-associated fluorescent pseudomonads, and it plays a key role in the suppression of a wide

variety of soil-borne diseases (Weller et al., 2002; de Souza et al., 2003a; Haas and Defago, 2005; Ramette et al., 2006). 2,4-DAPG inhibits zoopores produced by *Pythium* spp. and also damages the membrane of this Oomycetes (de Souza, 2003b). 2,4-DAPG-producing pseudomonads are of worldwide origin and are commonly found in the rhizosphere of important crops such as maize, pea, and wheat (Keel et al., 1996; Landa et al. 2002; Picard, 2000; Raaijmakers and Weller, 2001; Bergsma-Vlami et al., 2005a); they have also been shown as important biological components of the natural suppressiveness of certain agricultural soils to take-all disease of wheat (de Souza et al., 2003a; Weller et al., 2002), fusarium wilt of pea (Landa et al., 2002) and black root of tobacco (Ramette et al., 2006).

4.4.2. Pyoluteorin

Pyoluteorin (PLT) is a chlorinated polyketide antibiotic just like 2,4-DAPG (Fig. 1.1). Its production by several *Pseudomonas* species and its inhibitory activity against Oomycetes fungi including the plant pathogenic *Pythium ultimum* has been documented. For instance, the biocontrol agents, *P. fluorescens* Pf-5 and *P. fluorescens CHA0* suppressed *P. ultimum*-incited diseases through production of PLT, in addition to some other antibiotics (Bender et al., 1999). Apart from its established extracellular role as an antibiotic, PLT was also shown to function as an autoinducer and intercellular signal molecule which influences the spectrum of secondary metabolites produced by distinct populations of bacterial cells co-inoculating the rhizosphere. This was clearly demonstrated by Brodhagen et al. (2005), where PLT produced by co-inoculated *P. fluorescens* Pf-5 cells improved the expression of a pyoluteorin biosynthesis gene in a pyoluteorin-deficient mutant of *P. fluorescens* Pf-5.

4.4.3. Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl) pyrrole] (Fig. 1.1) is a broad-spectrum antifungal metabolite produced by strains of *Enterobacter agglomerans*, *Myxococcus fulvus*, *Corallococcus exiguous*, *Cystobacter ferrugineus, Serratia* spp. and several strains of *Pseudomonas* and *Burkholderia* (Hammer et al., 1999). The antibiotic was first isolated from *Pseudomonas pyrrocinia* (Hammer et al*.,* 1999). This highly active metabolite has been used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, and a phenyl pyrrol derivative of pyrrolnitrin has been developed as an agricultural fungicide (Dwivedi and Johri, 2003). Similarly in biocontrol studies, the role of pyrrolnitrin (PRN) in suppression of plant pathogens has been demonstrated by many investigators. Mutant strains of *P. fluorescens* BL915 deficient in PRN production had a greatly reduced ability to control *R. solani* seedling disease of cotton. Also transfer of a gene region that has a role in PRN synthesis derived from *P. fluorescens* BL915 into two *Pseudomonas* strains that were non-PRN producers nor effective antagonists of *R. solani,* conferred on the strains the ability to produce PRN and to antagonize *R. solani* in both *in vitro* and *in vivo* disease control assays with cotton (Hill et al., 1994). Pyrrolnitrin functions by inhibiting fungal respiratory chain (Tripathi et al., 1969).

4.4.4. Phenazine

Phenazines (Phz) are also antibiotics with broad-spectrum activity and they comprise a large family of over 100 compounds tricyclic ring nitrogen-containing brightly colored pigments (Fig 1.1) (Chin-A-Woeng et al., 2001; Kavitha et al., 2005). Currently, more than 50 naturally occurring Phz compounds have been described

and are exclusively produced by bacteria, such as *Pseudomonas, Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacteriu, Pantoea* and *Burkholderia* species (Turner and Messenger, 1986). Phenazine and its derivatives have been implicated in virulence, competitive fitness of the producing strains, and they are well-known for their antifungal properties (Mazzola et al., 2002; Chin-A-Woeng et al., 2001; Dwivedi and Johri, 2003). Production of Phz derivatives by a considerable number of *Pseudomonas* strains with antagonistic activity has been reported; most often is the production of pyocyanin, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) (Turner and Messenger, 1986; Chin-A-Woeng et al., 2000; Chin-A-Woeng et al., 2001; Haas and Defago, 2005). Production of PCA in *P. fluorescens* strain 2 – 79 and PCN in *P. chlororaphis* strain PCL1391 was reported as the major mechanism of suppression to take-all disease of wheat and tomato root rot, respectively (Slininger et al., 2000; Chin-A-Woeng et al., 1998). Biosynthesis of more than one Phz derivatives can occur simultaneously in many strains. For instance, simultaneous production of pyocyanin, PCA and PCN was found in *Pseudomonas aeruginosa* strains (Chang and Blackwood, 1969). The growth conditions influence the number and type of Phz synthesized by an individual bacterium (Dwivedi and Johri, 2003). Phz are redox active compounds, and thus, the mechanism for their action is assumed to be due to their ability to engage in redox cycling in the presence of various reducing agents and molecular oxygen, resulting in the accumulation of toxic superoxide ions, hydrogen peroxide (H_2O_2) which are harmful to the cell or can lead to the death of the cell (Mavrodi et al., 2001).

4.4.5. Antibiotic biosynthetic loci

The biosynthetic operons responsible for production of these four antibiotics have been identified and characterized in a number of *Pseudomonas* species using specific primers and probes (Raaijmakers et al., 1997; de Souza and Raaijmakers, 2003). 2,4-DAPG biosynthetic locus contained six open reading frames designated *phlEDBCAF* and are reported to be highly conserved among 2,4-DAPG-producing strains of *Pseudomonas* spp. The gene *phlD* is reponsible for the production of monoactylphloroglucinol (MAPG), a precursor of the 2,4-DAPG; whereas phlACB were found to be involved in the formation of 2,4-DAPG from MAPG; *phlE* produces a red pigment that is involved in the transport of 2,4-DAPG out of the cell (Bangera and Thomashow, 1999; Dwivedi and Johri, 2003).

The pyoluteorin biosynthetic gene cluster of *P. fluorescens* Pf-5 contains ten structural genes designated *pltLABCDEFG* and *pltMR* (a sequence within 486 bp intergenic region separates *pltMR* from the gene cluster) that are required for biosynthesis of PLT (Nowak-Thompson et al., 1999; Dwivedi and Johri, 2003).

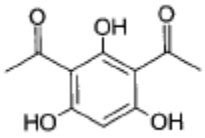
Chorismic acid is an important precursor of PCA while in some *Pseudomonas* spp. PCA acts as the key intermediate in the synthesis of other Phz derivatives. Genes responsible for the biosynthesis of the protein necessary for the conversion of chorismic acid to phenazine-1-carboxylic acid (PCA) were identified in *P. fluorescens* 2-79, *P. aeruginosa* PAO1 and *P. chlororaphis* PCL1319*;* it contained *phzABCDEFG* genes (Mavrodi et al., 2001; Chin-A-Woeng et al., 2002). Four genes (*prnABCD*) were found responsible for PRN biosynthesis (Hammer et al., 1999).

The isolation and characterization of these genes or gene clusters have greatly facilitated the primer design and the use of polymerase chain reaction (PCR) methods to rapidly detect the antibiotic genes (Raaijmakers et al., 1997; de Souza and Raaijmakers, 2003). Thus, identification, detection, characterization and

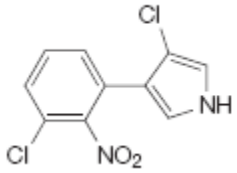
quantification of antibiotic producers, especially in a complex environment such as rhizosphere or bulk soil have been achieved.

None of the above discussed mechanisms are necessarily mutually exclusive and frequently several modes of actions are exhibited by a single biocontrol agent. For instance, *P. fluorescens* Pf-5 is notable as a biological control organism for the spectrum of antibiotics and other secondary metabolites that it produces. PRN, PLT and 2,4-DAPG are among the antibiotics produced by *P. fluorescens* Pf-5. It also produces hydrogen cyanide and the siderophores pyochelin and pyoverdin. Different combinations of these mechanisms are responsible for the inhibitory activity of *P. fluorescens* Pf-5 against different soil-borne pathogens (Paulsen et al., 2005). Trejo-Estrada et al. (1998) reported on a biocontrol agent, *Streptomyces violaceusniger* strain YCED-9 that antagonizes many different classes of plant pathogenic fungi. Factors responsible for the broad-spectrum antagonistic activity of the strain were production of three different anti-microbial compounds and two hydrolytic enzymes (chitinase and ß-1,3-glucanase). Additional information on the different mechanisms of activity displayed by biocontrol agents may be found in recent reviews (Whipps, 2001; Lugtenberg et al., 2002; Compant et al., 2005; Haas and Défago, 2005; Alabouvette, 2006).

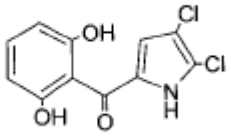
Key factors in the regulation of biosynthesis of several fungal metabolites, including those mentioned above are global regulation and quorum sensing. An example of a well-studied global regulatory system involved in the expression of biocontrol traits in many plant-beneficial gram-negative bacteria is GacS/GacA (Heeb and Haas, 2001), which is one of the subjects of the study presented in this thesis.



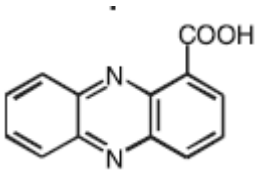
2,4-diacetylphloroglucinol



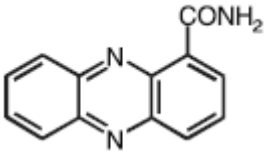
Pyrrolnitrin



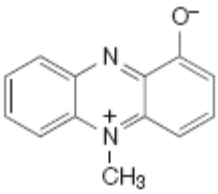
Pyoluteorin



Phenazine-1-carboxylic acid (PCA)



phenazine-1-carboxamide (PCN)



Pyocyanin

Figure 1.1. Structure of antibiotic compounds produced by biocontrol agents which have been implicated in pathogen inhibition {2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN)}.

5. The global regulatory system (GacS/GacA)

The sensor kinase GacS and the response regulator GacA are members of a two-component system that is present in a wide variety of gram-negative bacteria (Heeb and Haas, 2001). This regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA). It was proposed that GacS recognizes specific environmental stimuli and activates GacA, which in turn triggers the expression of specific genes (Appleby et al., 1996; Heeb and Haas, 2001). However, the exact substance that binds or activates GacS is still unknown. The environmental stimuli may be abiotic or biotic; the abiotic stimuli include temperature, osmolarity, and pH. Whereas the biotic stimuli are produced by the host bacteria or by the bacterial population when sufficient cell densities are reached, and thus regulate quorum sensing (Heeb and Haas, 2001). The term quorum sensing is used to describe the phenomenon of extracellular cell-to-cell communication of many bacteria through emittance and sensing of chemical signals (e.g. N-acyl-homoserine lactones, AHLs; autoinducer, AI-2) as their cell densities increase (Dubuis and Haas, 2007). In *P. aeruginosa*, the GacS/GacA system modulates the quorum sensing response by regulating AHL synthesis (Kay et al., 2006).

Full expression of genes involved in virulence factors of plant or animal pathogenic bacteria are controlled by the GacS/GacA system (Cui et al., 2001). Similarly, in plant beneficial bacteria, biosynthesis of secondary metabolites required for biocontrol activity, such as 2,4-DAPG, Phz, PLT, and PRN, as well as hydrogen cyanide, chitinase, and exoproteases are directly regulated by the GacA/GacS system (Chancey et al., 1999; Aarons et al., 2000; Duffy et al., 2000). Global activator of

antibiotic and cyanide (GacA) gene (*gacA*) was first described for *Pseudomonas fluorescens* CHA0, a biocontrol strain with broad-spectrum activity against many plant pathogens (Laville et al., 1992). The antifungal activity of CHA0 is a result of its ability to produce many secondary metabolites including 2,4-DAPG, Phz, PLT, and PRN, hydrogen cyanide, and exoproteases. The ecological fitness of this strain and the genes responsible for the production of these metabolites are directly regulated by the Gac/GacS system (Laville et al., 1992; Heeb and Haas, 2001).

GacS- and GacA-negative mutants in some strains have been described as being signal blind to stimuli recognized by the GacS/GacA system. Thus expression of various genes involved in the ecological fitness and secondary metabolites production by the biocontrol wild-type strains was negatively regulated, resulting in abolishment of biocontrol activity (Laville et al., 1992; Heeb and Haas, 2001). For instance, Siddiqui et al. (2005) demonstrated reduction in the biological control activity of a mutant strain of *P. fluorescens* CHA0 that is deficient in *gacA* gene. Introduction of *gacS* gene into mutant strains of *P. fluorescens* PfG32R was shown to restore protease activity and antifungal activity against *F. oxysporum*. However, the antibacterial activity and siderophores production were maintained in the GacS deficient strains of *P. fluorescens* PfG32R, indicating that these two activities were not controlled by the GacS/GacA system (Alit-Susanta and Takikawa, 2006). In another experiment, Ge et al. (2004) demonstrated differential regulation of the production of phenazine-1-carboxylic acid (PCA) and PLT by *gacA* gene in the biocontrol agent, *Pseudomonas* sp. M18. While the latter was reduced to non-detectable level in the *gacA* insertional mutant strain M18G, the former was increased by 30-fold. A list of phenotypes controlled by the *GacS/GacA* system in various gram-negative bacteria was compiled by Heeb and Haas (2001). About 20

GacA/GacS homologs have been identified in gram-negative bacterial genera such as *Pseudomonas* strains, *Vibrio cholerae*, *Azotobacter vinelandii*, and many enteric bacteria including *Escherichia coli*, *Salmonella enterica* serovar *typhimurium* and *Erwinia carotovora* spp. *carotovora.* Alignment of 16 GacA proteins of eight *Pseudomonas* strains, and eight other strains of *Xylella, Shewanella* and the enteric bacteria, showed that the eight *Pseudomonas* strains clustered together and separated distantly from other strains (Heeb and Haas, 2001), suggesting that the *gacA* gene may be conserved within the genus *Pseudomonas.* The design of the primers targeting the *gacA* gene in *Pseudomonas* spp. confirmed that the gene is highly conserved within the genus *Pseudomonas* (de Souza et al., 2003c). Thus, these authors proposed the use of *gacA* as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples. Chapter 4 of this thesis presents data on the diversity of *gacA* genes in a collection of antagonistic *Pseudomonas* spp. using a novel PCR-DGGE fingerprinting method.

6. Soil-borne pathogens

There are large numbers of bacteria and fungi and some other microorganisms inhabiting the soil. These organisms are important for maintaining the fertilility of the soil. Nonetheless, some may be pathogenic to plants, attacking the plant roots and causing diseases; they are referred to as soil-borne pathogens. These pathogenic microorganisms are harmful to the plants and they cause various diseases in crops such as root and crown rots, vascular wilting, take-all and damping-off. The extent of their harmful effects ranges from mild symptoms to catastrophes where large fields planted with agricultural crops are destroyed. Thus, they are major and chronic threats to food production and ecosystem stability

worldwide. Loss of at least 10% of global food production to plant diseases aggravates the deficit of food supply in which at least 800 million people are inadequately fed (James, 1998; Christou and Twyman, 2004). Examples of such soil-borne plant pathogens in the fungal kingdom are *Fusarium oxysporum* and *Rhizoctonia solani*; and in the bacterial kingdom is *Ralstonia solanacearum,* which are part of the subjects of this thesis.

6.1. *Fusarium oxysporum*

Fusarium oxysporum is an imperfect fungus (one with no known sexual stage) belonging to the Ascomycetes. Three types of asexual spores are produced by the fungus: microconidia, macroconidia, and chlamydospores (Agrios, 1988). The most abundant and commonly produced spores under all conditions are microconidia, which are one- or two-celled. Macroconidia are three- or more celled, with pointed and curved ends; they are frequently seen on the surface of infested plants killed by the fungus (Gonsalves and Ferreira, 1993). Micro- and macroconidia are produced for short-term survival and dispersal of the fungus. Chlamydospores are viable, thick-walled spores, filled with lipid-like material; they are the only spores produced for long-time survival in the soil when the host plant is not available (Agrios, 1997; 1988). *F. oxysporum* is described as having cosmopolitan distribution (Gonsalves and Ferreira, 1993); they include not only plant pathogenic and human pathogenic strains but also non-pathogenic strains (Burgess, 1981; Boutati and Anaissie, 1997). The antagonistic potential of the non-pathogenic strains towards various pathogenic *F. oxysporum* has been extensively studied; and natural suppressiveness to Fusarium wilts has been attributed to their dominance in suppressive soils (Mandeel and Baker, 1991; Alabouvette and Couteaudier, 1992; Alabouvette et al., 2001). The

non-pathogenic isolates of *F. oxysporum* do not cause any disease symptoms, despite their efficient colonization of plant roots (Olivain and Alabouvette, 1999; Paparu et al., 2006).

Certain members of the pathogenic strains of *F. oxysporum* have been reported as serious human pathogens causing diseases such as hematologic cancer (Boutati and Anaissie, 1997). The plant pathogenic *F. oxysporum* strains cause diseases in agricultural plants resulting in loss of yield (Fravel et al., 2002). The fungus also produces poisonous chemical compounds, i.e. “mycotoxins” which contaminate harvested crops. Ingestion of crops contaminated with these toxins may give rise to allergic symptoms or be carcinogenic by long-term consumption in humans and animals (Nelson et al., 1994). These toxins may also operate in nature to disable plant defense mechanisms or to defend the fungus against other microorganisms. Fusaric acid, a mycotoxin produced by strains of *F. oxysporum* was found to repress the production of 2,4-DAPG in the biocontrol strain, *P. fluorescens* CHA0 (Notz et al., 2002). Mycotoxins produced by *F. oxysporum* also include diacetoxyscirpenol, T-2 toxin and zearalenone (Chakrabarti and Ghosal, 1987; Notz et al., 2002). The pathogenic strains of *F. oxysporum* are grouped into several specialized forms - known as formae speciales (f. sp.) – on the basis of their ability to cause disease on similar or identical host range; they are divided into more than 120 different formae speciales (Armstrong and Armstrong, 1981). Some of the formae speciales are further divided into subgroups, named races, on the basis of virulence to a set of differential cultivars within the same plant species (Armstrong and Armstrong, 1981). Diseases caused by *F. oxysporum* and its several formae speciales on agricultural crops include rot of roots, root crowns, vascular wilts and post-harvest decay. Vascular wilt is the most common and serious disease caused by *F. oxysporum* on

many different plant species (Andre et al., 1993). The mode of the pathogen infection is by invading the plant roots through natural wounds, nematode feeding punctures, and other wounds. After penetration it travels through the xylem of the plant that moves water and nutrients from the root to the crown of the plant. The mycelia produce toxic substances as they ramify through the plant, eventually the xylem becomes obstructed and the plant wilts and dies (Pietro et al., 2003). It is difficult to generalize the fungus condition of infection. Most formae speciales grow in soil at any degree of moisture favorable for their host crops. However, as it is for most Fusarium wilt, low soil moisture favors the pathogen and aggravates the wilting symptom. The optimum temperature of infection for most pathogenic strains ranges between 27-28^{o}C (Armstrong and Armstrong, 1981). Slightly acidic pH (pH 5-5.5), high nitrogen, low calcium, and low potassium levels in the soil can favor disease development (Zitter, 1998). The management of pathogenic *F. oxysporum* is difficult because of its wide host range and ability to grow saprophytically (on dead organic matter such as crop wastes) or survive for extended periods in the form of thick-walled chlamydospores in the absence of a susceptible crop (Agrios, 1997; Pietro et al., 2003). Use of resistant varieties is the only practical measure for controlling the disease in the field (Tamietti and Valentino, 2006). Breeding for resistant varieties may pose a problem, especially if no dominant gene is known (e.g flax). Also new races of the pathogen can emerge which possess host resistance (Fravel et al., 2002). Under greenhouse conditions, soil sterilization can be performed (Tamietti and Valentino, 2006), however, rapid disease spread can occur if the sterilized soil is re-contaminated by the fungus, in addition, it is far too expensive for most farmers.

6.2. *Rhizoctonia solani*

Rhizoctonia solani is a basidiomycete fungus, which does not produce any asexual spore (called conidia) but can produce sexual spores (basidiospores) occasionally (Ceresini, 1999). In nature, *R. solani* reproduces asexually and exists primarily as thin, vegetative strands called hyphae. They also produce specialized hyphae composed of compact cells called monilioid cells, which fuse together to produce hard structures called sclerotia. The monilioid cells are resistant to environmental extremes, allowing the fungus to survive adverse conditions. Isolates of *Rhizoctonia solani* are genotypically diverse. Anastomosis groups or AG subgroups have been used for identification and classification purpose. The isolates are identified and classified into anastomosis groups (AGs) according to the ability of their hyphae to anastomose (fuse) with one another (Anderson et al., 1982). To date, isolates of *R. solani* have been assigned to 14 AGs, including AG-1 to AG-13 and AG-B1 (Anderson et al., 1982; Carling et al., 2000). While four of the fourteen AGs are not pathogenic (AG6,7,10 and AG BI), four (AG-1, -2, -3, and -4) cause important diseases on plants worldwide; and the remaining AGs are less destructive pathogens with generally more restricted geographic distributions (Carling et al., 2002). Isolates within AGs tend to have similar host ranges; according to their morphology and pathogenicity they have been classified into subgroups: four subgroups of AG-1, eight of AG-2, 3 of AG-4, two of AG-6, five of AG-8, and two of AG-9 have been reported (Carling, 2000; Priyatmojo et al., 2001; Fenille et al., 2002). Four subgroups of AG1 are AG1-IA, AG1-IB, AG1-IC and AG-ID (Priyatmojo et al., 2001). The two AGs that are of interest in the study presented in this thesis are AG1-IB and AG3 (Chapters 3 and 5). Isolates in AG1-IB cause bottom rot or rot of cabbage and lettuce plants and many of them cause web blight in grains, while isolates of AG3, which

cause stolon/stem cankers and black scurf on potato tubers are mainly pathogens of potato (Anderson et al., 1982). Rhizoctonia-incited diseases on a wide range of grain, vegetables, and fruit crops worldwide also include seed decay, damping-off of seedlings, root rot, pod rot, fruit decay and aerial blight (Ceresini, 1999). The infection process of the fungus, after gaining entry into the plant cell wall, is by the production of many different extracellular enzymes that degrade various components of plant cell walls (e.g. cellulose, cutin and pectin). Cool temperature, high soil fertility, high soil moisture, pH of 7 or less and soil temperatures of 12-32 ℃ favor the disease development.

Aggressive isolates of *R. solani* are difficult to control because of a number of intrinsic properties such as the wide host range, large sclerotia being insensitive to fungistasis and resistant to decomposition, rapid colonization of fresh organic matter, extensive mycelial growth, mycelium of high biphenolic content that is relatively resistant to degradation, hyperparasitic potential, and capacity to escape soil competition under humid conditions by growing on surface organic matter or aerial plant-to-plant spread (Papavizas, 1970).

6.3. *Ralstonia solanacearum*

Ralstonia solanacearum (previously named *Pseudomonas solanacearum*) (Yabuuchi et al., 1995) is a soil-borne pathogen which causes wilting disease in plants and is a member of the Proteobacteria *β* subdivision. The bacterium is considered as one of the most important plant pathogenic bacteria because of its broad geographical distribution in tropical, subtropical and warm regions of the world and extensive unusual host range of more than 50 plant families (Hayward, 1991; Hayward, 2000).

The host range includes solanaceous plants (e.g. tomato, potato, tobacco, eggplant), some leguminous plants (such as groundnut, French bean), a few monocotyledons (mainly banana, ginger) and several tree and shrub hosts (e.g. mulberry, olive, cassava, eucalyptus) (Genin and Boucher, 2002). Based on the host(s) affected, *R. solanacearum* have been classified into five races and grouped into five biovars according to their ability to utilize and/or oxidize several hexose alcohols and disaccharides (Hayward, 1991).

Race 1 has the widest world distribution on a range of economically important hosts, race 2 on banana and other *Musaceae*, and race 3 on potato and other *Solanaceae* species (CABI/EPPO, 1990; Elphinstone, 2006). According to the restriction fragment length polymorphism (RFLP), isolates of the bacterium have been divided into three major divisions, one primarily of Asian origin, the second from America and the third division recently identified is of African origin, indicating a distant evolutionary dichotomy (Cook et al., 1989; Poussier et al., 2000). Due to this high phenotypic and genotypic variation among isolates of *R. solanacearum,* diseases caused by this bacterium are difficult to control (Genin and Boucher, 2002). *R. solanacearum* penetrates plant roots through wounds or at sites of secondary root emergence; for some strains aerial transmission by insects has also been documented (Genin and Boucher, 2002). After root invasion, the bacterium colonizes the intercellular spaces of the root cortex and vascular parenchyma; then it spreads rapidly to the aerial parts of the plants through the xylem system. The vascular abnormality induced as a result of this extensive colonization leads to wilting symptoms and eventual plant death. The bacterium is able to survive in the soil for long periods in the absence of host plants due to the association of the bacterium with plant debris or with several weed hosts that are symptomless carriers (Hayward,

1991). In addition, like many other soil microbe, the *R. solanacearum* is capable of entering a dormant-like 'viable but not culturable' state (Grey and Steck, 2001).

Bacterial antagonists with both antifungal and antibacterial activity can be used for dual biocontrol purposes to combat plant diseases caused by soil-borne bacteria and fungi. Thus, bacteria with anti-fungal activity isolated in this study were further screened for their ability to inhibit bacteria; *R. solanacearum* was chosen as a model gram-negative bacterium. This screening also serves as indication of antibiotic production (Chapter 3).

7. Searching for biocontrol agents

The ultimate success of biocontrol depends on how well the searching and screening process is done. There is no single, distinct way to search or screen for biocontrol agents. The searching and screening for biocontrol agents depend on the target pathogen, the crop and the cropping system (Fravel, 2005). Insights as to where to look for antagonists were provided by Cook and Baker (1983), Schisler and Slininger (1997) as well as other investigators. Isolation of biocontrol strains from the same environment in which they are to be used may enhance the chance of selecting effective biocontrol agents. For this reason, widely used approaches to select for potential biocontrol agents include isolation of antagonists from soils that are naturally suppressive to a particular pathogen (suppressive soils), and isolation from intended environment of use, such as soils, seeds or roots. Considering these two approaches, biocontrol agents have been isolated from different environments, most especially from suppressive soils, agricultural soils, compost-amended soils, rhizosphere, endosphere, spermosphere, phyllosphere (Larkin et al., 1996; Cattelan et al., 1999; Hoitink and Boehm, 1999; Martunez et al., 2002; Nair et al., 2002;

Cavaglieri et al., 2004; Islam and Toyota, 2004; Berg et al., 2005). Even in environments such as marine sediments and marine water, bacterial antagonists have been isolated for biocontrol purpose in aquaculture (You et la, 2005). Biological control organisms investigated here were isolated from suppressive and agricultural soils.

7.1. Suppressive soils as sources of antagonistic microorganisms

Soils possess a vast and diverse microbial population, including both bacteria and fungal community. Some soils are naturally inhospitable to plant pathogens by limiting either their survival or growth. Such soils are regarded as pathogen- or disease-suppressive soils (Garbeva et al., 2004). Soils suppressive to plant pathogens have also been defined by Baker and Cook (1974) as those in which a specific pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil. Furthermore, the concept of disease suppression has been defined in terms of “general” and “specific” suppression. Every soil has natural potential to suppress the activity of plant pathogens to some degree due to the presence and activity of soil microorganisms. This phenomenon is termed “general suppression” or "non-specific antagonism" or "biological buffering" (Weller et al., 2002). It is assumed to be related to the total microbial population (biomass) in a given soil, which competes with the pathogen for available resources or causes direct forms of antagonism through inhibition rather than operating through the action of a specific microorganism and specific group of microorganisms. General suppression can be enhanced by certain agronomical practices, addition of organic matter, or the build-up of soil fertility or any other

activity that leads to increase soil microbial activity. One specific population of microorganism is not responsible for general suppression and the suppressiveness is not transferable between soils (Weller et al., 2002; Haas and Defago, 2005). In contrast, activities of one or several specific populations of microorganisms might be responsible for specific suppression (Weller et al., 2002; Mazzola, 2002). Natural disease suppression observed in suppressive soils results from a combination of general and specific suppression. The two function as a continuum in the soil, though they may be affected differently by abiotic factors (climatic, edaphic, and agronomic conditions) (Weller et al., 2002).

Even though, there are plentiful evidences for the role of both abiotic (soil physico-chemical properties) and biotic (soil microflora) elements of the soil in disease suppression, soil suppressiveness is often directly or indirectly a function of the activity of soil microorganisms or microbial metabolites (Murakami et al., 2000; Alabouvette, 1986; Mazzola, 2002; Weller et al., 2002). The role of microorganisms in disease suppression has been confirmed by several studies, as disease factors were eliminated through soil fumigation or heat-sterilization, or steam pasteurization and gamma irradiation; and re-introduction of the pathogen results in an increased disease severity (Weller et al., 2002). Transfer of disease suppressive potential by application of disease suppressive soils to disease-conducive soils provided further evidence for the importance of microorganisms in natural disease suppression (Weller et al., 2002). One good example is the specific suppression associated with take-all disease of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* that is eliminated by treating the soil with moist heat (pasteurization, 60 ℃ for 30 min); and it is transferrable by adding 1-10% take-all suppressive soil to raw

conducive, fumigated, or pasteurized soil (Andrade et al., 1994; Raaijmakers and Weller, 1998).

Naturally disease-suppressive soils occur worldwide and have been reported for many soil-borne plant pathogens (Cook and Bakker, 1983), including the following pathogens (examples of associated diseases are given in parentheses): *Gaeumannomyces graminis* var. *tritici* (take-all of wheat) (Cook and Rovira, 1976); *Fusarium oxysporum* (wilt diseases of several crops) (Alabouvette, 1986); *Plasmodiophora brassicae* (clubroot of cabbage and related crucifers) (Murakami et al., 2000; Worku and Gerhardson, 1996); *Pythium* spp. and *Rhizoctonia solani* (damping-off and rot of seedlings of several crops) (Mazzola, 1999; Garbeva et al., 2004); *Streptomyces scabies* (bacterial potato scab) (Lorang et al., 1995; Liu et al., 1996); *Ralstonia solanacearum* (bacterial wilt of tomato, tobacco and other crops) (Hayward, 1991).

With the overwhelming evidences that disease suppression in naturally occurring disease suppressive soils is due to the activities of microorganisms, many authors have proposed that suppressive soils are reservoir of natural, effective and valuable microbial antagonists, and the chance of selecting effective biocontrol strains might be improved if isolations were made from such soils (Weller, 1988; Cook, 1990). In addition such systems can serve potentially as models for understanding the mechanisms by which complex plant-associated microorganisms interfere with plant pathogenesis. As a result, during the past 20 years, many biocontrol organisms were isolated from soils naturally suppressive to certain soil-borne plant pathogens.

8. Efficacy of biocontrol inoculants: rhizosphere competence and non-target effects

Application of microbial antagonists as inoculants to suppress plant pathogens is very appealing and its potential for sustainable agriculture has been reviewed by some authors (Haas et al., 2000; Bloemberg and Lugtenberg, 2001; Haas and Defago, 2005; Mark et al., 2006). Nonetheless, for many microbial antagonists, lack of correlation between *in vitro* antagonistic activity and field performance as well as inconsistent and inefficient biocontrol in diverse field situations have been reported as the main drawback of the approach (Schottel et al., 2001; Faltin et al., 2004; Kiely et al., 2006). Consequently, only few strains have been commercialized as biopesticides and biofertilizers. Biocontrol of pathogen is a multi-trait phenomenon which success relies on many factors. Some of the factors are external such as edaphic and climatic conditions but the main factor is the inherent traits of the biocontrol strains, which include sufficient production of required metabolites or efficient root colonization (rhizosphere competence) (Mark et al., 2006). Colonization of a large part of the root system by biocontrol agent is required for efficient suppression of pathogen because it is believed to function as the delivery system for the cells producing the anti-fungal metabolites (Lugtenberg et al., 2001). Furthermore, the expression of genes involved in biosynthesis of most extracellular metabolites are cell-density dependent, such that the production of the metabolites is enhanced above a certain population threshold (Haas and Defago, 2005). Bull et al. (1991) reported an inverse relation between the numbers of bacteria present on the wheat root and the numbers of take-all lesions seen on the plant. In contrast, Roberts et al. (1994) claimed that colonization is less significant for biocontrol. Nonetheless,

with the use of a series of different genes involved in root colonization, several authors have demonstrated that rhizosphere competence plays a crucial role in determining the success of biological control agents (Lugtenberg et al., 2001). For instance, Chin-A-Woeng et al. (2000) clearly demonstrated that a mutant of *P. chlororaphis* PCL1391, impaired in colonization traits only, was no longer able to protect tomato from foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici.* Rhizosphere competence of biocontrol agents (BCAs) comprises aggressive root colonization coupled with the ability to survive, proliferate and maintain a minimal population threshold along growing plant roots over a considerable time period, in the presence of the indigenous microflora. In addition, the BCAs should be able to protect the plant host at both infectious time and site that are favorable for root infection by the pathogens (Chin-A-Woeng et al., 2000; Validov et al., 2005).

In addition to effective disease suppression promoted by good rhziosphere competence, another necessary condition for introducing a biocontrol agent into the environment is that the effects of the introduced BCAs on non-target organisms should be at least tolerable, if not negligible. Adverse effects of the introduced BCAs on any other organisms rather than the target organism is referred to as non-target effects (Winding et al., 2004). Most BCAs produce secondary metabolites with antifungal activity and their effects are not necessarily restricted to root pathogens. For example, 2,4-DAPG and PLT have also been reported to affect other soil microorganisms, including bacteria (Natsch et al., 1998), protozoa (Schlimme et al., 1999) and fungi (Girlanda et al., 2001). Another non-target effect may be competition with the indigenous microorganisms, for nutrients and space when applying large numbers of antagonistic strains for biocontrol, especially microorganisms closely

related to the BCA itself (Natsch et al., 1998; Moënne-Loccoz et al., 2001). The community of resident microorganisms in the rhizosphere plays a key role in the functioning of the ecosystem through its contribution to plant health, nutrient cycling, and soil fertility (Moënne-Loccoz et al., 2001; Winding et al., 2004). It is therefore important to understand how biocontrol inoculants can influence the structure of the non-target microbial community within the agricultural ecosystem.

8.1. Methods to assess the survival, rhizosphere competence and non-target effects of biocontrol inoculants

Several methods have been used to study the rhizosphere competence and fate of biocontrol inoculants in the rhizosphere of inoculated plants, most of which were recently reviewed by Gamalero et al. (2003). These methods include culture-dependent and -independent methods. Dilution plating (culture-dependent method) is the most basic method used in most studies to quantify inoculant and bacterial density by colony counts. One of the drawbacks in the use of culture-dependent methods to monitor BCAs is the phenotypic similarity among some microorganisms living in the soil, which may pose difficulty in differentiating the strain released into the soil from the indigenous strains. To overcome this drawback at the strain level, most colonization studies have used antibiotic-resistant mutants (e.g. rifampicin resistant) of the wild-type strain (Lottmann et al., 2000; Moënne-Loccoz et al., 2001). Another limitation of the plating method is underestimation of the actual population size because bacteria could enter in a viable but non-culturable (VBNC) state. The VBNC state depicts a transient loss of ability to grow and develop colonies on nutrient medium, while still being metabolically active (Oliver, 2000). This state has been reported for several enteric bacteria (McDougald et al., 1998; Pujol et al.,

2006). The VBNC state has also been reported for *P. fluorescens* CHAO, the biocontrol agent of several soil-borne diseases (Marscher et al., 2000). As a result of these drawbacks, representativeness of the real processes in soil based on the data obtained with culture-dependent methods has been questioned. In spite of these drawbacks, culture-dependent methods remain widely used, mainly because the methods are less costly, easy to apply and still of great significance in providing information on the morphology of inoculated strains.

In addition to dilution plating, culture-independent methods are used in monitoring survival of inoculated antagonistic bacteria (Chapter 5 of this dissertation). In contrast to the culture-dependent approach, monitoring of specific generic target in the soil or rhizosphere is possible with culture-independent methods. These methods also give a dynamic and broader picture of the total microbial population regardless of their cultivability (Gamalero et al*.,* 2003). The detection method is based on polymerase chain reaction (PCR) amplification of nucleic acids extracted from the soil or the rhizosphere or any environmental sample. The 16S and 18S ribosomal RNA (rRNA) or their rRNA genes represent useful ecological markers for prokaryotes and eukaryotes, respectively. PCR has been used in some form for detection of bacterial inoculants in environmental samples, such as the most probable number PCR (MPN-PCR), the competitive PCR (C-PCR) and quantitative PCR (Q-PCR) (Gamalero et al*.,* 2003). Recently, Pujol et al. (2006) used the real time PCR (RT-PCR) to detect and quantify bacterial inoculants in environmental samples. In order to study the effect of the inoculated strains on the microbial community, some investigators have also combined PCR with other techniques. A good example is PCR-denaturing gradient gel electrophoresis (DGGE), a molecular fingerprinting technique that is useful for monitoring the survival of inoculated strains in the rhizosphere or soil and

to determine their effect on the genotypic structure of the indigenous microbial communities (Lottmann et al., 2000). However, the monitoring of the inoculants' survival with this method is only possible when they belong to the dominant microbial communities. In addition, with this method the composition, diversity and changes in bacterial or fungal communities in a wide range of environmental samples can be assessed (Muyzer and Smalla, 1998). As good as these methods are, they also have their limitations, which include inability to differentiate between viable and non-viable cells (Gamalero et al., 2003).

Due to the limitations associated with culture-dependent and -independent methods, a polyphasic approach that combines both methods and in some cases with other methods such as confocal laser scanning microscopy (CLSM) have been used by some workers. This has provided valuable insights into the survival, colonization patterns of the inoculants, and their influence on the diversity of the microbial populations in the rhizosphere of the inoculated plants. In a recent study, Götz et al. (2006) evaluated the survival and colonization patterns of two gfp-tagged BCAs (*P. putida* PRD16 and *Enterobacter cowanii* PRF116) in the rhizosphere of tomato plants over three weeks and the effects of their inoculation on the indigenous bacterial community using selective plating, molecular fingerprinting (PCR-DGGE), and CLSM. Decrease in relative abundance of the two gfp-tagged antagonists observed by selective plating was also confirmed with analysis of 16S rRNA gene fragments amplified from total community DNA by DGGE and CLSM. With PCR-DGGE the authors were able to assess the influence of the inoculants on the indigenous bacterial population as revealed by two dominant bands, which were present in all treatments and at all sampling times; and one band that was not detected in the replicates of the inoculated samples but appeared in the control. van

Overbeek et al. (2002) employed a similar polyphasic approach to study the behaviour of a known strain of *R. solanacearum* in bulk soil and in soil from the tomato rhizosphere. Fluorescent *in situ* hybridization (FISH) assays were used to demonstrate differences in root colonization by the pathogen following treatment with strains of a potential biocontrol agent, *P. corrugata*. PCR was also combined with denaturing gradient gel electrophoresis (DGGE) analysis to support evidence for antagonism between *P*. *corrugata* and *R. solanacearum*. Strong bands were seen in soil profiles from systems containing only the pathogen, whereas weak bands of *R. solanacearum* were detected in profiles from mixed systems. For further confirmation, the authors also used viable counts, *gfp* as a genetic marker, to trace the spread of the known strain of *R*. *solanacearum*. This use of multiple detection methods confirmed and validated results obtained by the molecular assays. In this thesis, dilution plating was supplemented with molecular fingerprinting methods (PCR-DGGE analysis) to assess the survival and rhizosphere competence of inoculated BCAs, and their non-target effects on the indigenous bacterial and fungal communities.

OUTLINE OF THE THESIS

The chance of selecting effective biocontrol agents may be improved by isolating biocontrol strains from the same environment in which they are to be used. In disease-suppressive soils suppression is achieved without the use of chemicals and has been correlated with the presence of increased number of antagonistic bacteria in the soils (Weller et al., 2002). Thus, disease suppressive soils are considered as reservoir of antagonistic microorganisms. In this thesis four soils with documentation of disease suppression (specific suppression), originating from different locations in Europe were selected for study: France (FR), the Netherlands (NL), Sweden (SE) and the United Kingdom (UK). FR is suppressive to *Fusarium oxysporum*, NL to *Rhizoctonia solani* AG3, SE to *Plasmodiophora brassicae* and UK to *Gaeumannomyces graminis*. Knowing that every natural soil possesses a degree of disease suppression to plant pathogens (general disease suppression), two additional soils without documentation of disease suppression were also selected from two sites in Germany, Berlin (G-BR) and Braunschweig (G-BS).

The work presented in this dissertation contributed to an EU-funded project entitled *"Soil metagenomics to identify novel mechanisms of antagonism and antifungal activity for the improved control of phytopathogens (METACONTROL)".* Adesina M.F. (the doctoral candidate) received study grant from German Academic Exchange Service (DAAD).

The aims of the study presented in this thesis as described in Chapters 3-5 are as follows:

Chapter 3

The objectives of the study presented in Chapter 3 were to determine whether

(1) the proportion and diversity of bacteria with antagonistic properties towards *R. solani* and *F. oxysporum* differ between soils with different history of suppressiveness,

(2) particular antagonistic attributes can be related to a given soil type, functioning as a potential indicator of major antagonistic mechanisms that confers suppressiveness to these soils.

To achieve these objectives, the abundance and composition of bacterial antagonists towards *F. oxysporum* and *R. solani* was determined by isolating dominant culturable bacteria from the different soils on R2A. In addition, plating onto *Pseudomonas* (KMB+) and *Streptomyces* (AGS) selective media was done because these bacterial genera are frequently reported as *in vitro* antagonists. Isolates with antifungal activity were further screened for antibacterial activity as indicator of antibiotic production. *R. solanacearum* (a causal agent of bacterial wilt in many crops) and *B. subtilis* were used as model gram-negative and -positive bacteria, respectively. Moreover, the antagonists were phenotypically characterized for possible mechanisms of antagonistic activity based on glucanase, cellulase, protease, chitinase and siderophore production. Most of the antagonistic isolates were identified by FAME and/or 16S rRNA gene sequencing.

Chapter 4

The production of 2,4-DAPG, phz, PRN and PLT by several strains has been implicated in the suppression of soil-borne pathogens and is regulated by two-component system GacA/GacS in some gram-negative bacteria, particularly, the genus *Pseudomonas*. The design of the primers targeting *gacA* gene in *Pseudomonas* spp, showed that the gene is highly conserved within the genus *Pseudomonas.* Thus, the use of *gacA* gene as a complementary genetic marker for detection of *Pseudomonas* in environmental samples was proposed (de Souza et al.,

2003c). Likewise, with the development of a novel PCR-DGGE system, the detection of *gacA* gene in the soil has been achieved. Following this line of research, the aims of the study presented in Chapter 3 were

(1) to screen the collection of *Pseudomonas* antagonists obtained from Chapter 3 for the presence of the genes involved in biosynthesis of four antibiotics (2,4-diacetylpholorglucinol, *phlD;* pyrrolnitrin, *prnD;* pyoluteorin, *pltC;* and phenazine, *phzCD*, which are often regulated by GacS/GacA system),

(2) to determine the diversity of the *gacA* gene among the *Pseudomonas* antagonists retrieved from each soil using PCR-DGGE analysis and to assess the discriminating power of this system with BOX-PCR fingerprinting,

(3) to determine whether *Pseudomonas* isolates that possess similar *gacA*-DGGE mobilities also share common antagonistic traits (i.e. relationship between the different *gacA* genotypes and antagonistic function),

(4) to determine whether the different culture-derived *gacA* DGGE types can be linked with the *gacA* community DGGE profile of their corresponding soil of isolation.

To achieve these aims, all antagonistic bacterial isolates retrieved from the six soils (in Chapter 3), which belong to the genus *Pseudomonas*, were screened by PCR-Southern blot hybridization for antibiotic genes (*phlD, prnD, pltC* and *phzCD*). Diversity of the *gacA* gene among the antagonistic *Pseudomonas* spp was determined using DGGE. The discriminating power of this system was assessed using BOX-PCR fingerprints generated from genomic DNA of isolates. Cultivation-dependent and cultivation-independent methods were linked by determining whether the DGGE ribotypes of *gacA* gene derived from the collection of culturable

Pseudomonas antagonists corresponded to the dominant *gacA* DGGE ribotypes obtained from the community DNA of their respective soil of isolation.

Chapter 5

It is relatively easy to isolate a large number of potential biocontrol candidates in an *in vitro* assay. However, many studies have reported variability in the performance of biological control agents and lack of correlation between *in vitro* inhibitory activity and field performance of microbial antagonists. Therefore, rhizosphere competence and disease suppressive potential of *in vitro* antagonists need to be evaluated in growth chamber or greenhouse experiments that mimic field conditions in order to ascertain their efficacy. The non-target effects are also another crucial factor to be considered before a field release. For these reasons the aims of the study in Chapter 5 were

1. to evaluate the efficacy of some selected *in vitro* bacterial antagonists to control bottom rot disease of *R. solani* AG1-IB on lettuce plants,
2. to determine their rhizosphere competence, survival and growth promoting effects on lettuce plants,
3. to monitor the non-target effects of the most promising BCA on the indigenous bacterial and fungal communities.

In order to achieve these aims, ten of the antagonists generated in Chapter 3 were evaluated in growth chamber experiments for their ability to control bottom rot disease caused by *R. solani* AG1-IB on lettuce plant. They were selected based on strong *in vitro* activity against *R. solani* (AG3 and AG1-IB) or dual activity against *R. solani* (AG3 and AG1-IB) and *F. oxysporum*, and their ability to colonize lettuce plants in a pre-screening greenhouse experiment. In the growth chamber experiments the survival of the antagonists, disease severity in inoculated plants and colonization efficiency of the antagonists were assessed. The four best antagonists

were also evaluated for their growth promoting effects on lettuce plants. For the most promising biocontrol strain, the survival in the rhizosphere and its influence on the non-target indigenous total bacterial, specific bacterial (16S rRNA-specific and *gacA*-specific *Pseudomonas* community) and fungal communities were further monitored using a cultivation-independent approach that is based on PCR-DGGE fingerprinting techniques.

Chapter 6

In Chapter 6 general discussion and conclusion on the results obtained in Chapters 3-5 are presented.

CHAPTER 3

CHAPTER 3

Screening of bacterial isolates from various European soils for antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site dependent composition and diversity revealed

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Abstract

A cultivation-based approach was used to determine the *in vitro* antagonistic potential of soil bacteria towards *Rhizoctonia solani* AG3 and *Fusarium oxysporum* f. sp. *lini* (Foln3). Four composite soil samples were taken from six agricultural sites, which originated from France (FR), the Netherlands (NL), Sweden (SE), the United Kingdom (UK), and two locations in Germany, Berlin (G-BR) and Braunschweig (G-BS). Four of the soils (FR, NL, SE, UK) were previously reported as suppressive towards plant diseases while the two soils from Germany had no documentation of disease suppression. Serial dilutions from the composite soil samples were plated on R2A, AGS and King´s B media. A total of 1,788 isolates (approximately 100 isolates per medium and site) was screened for antifungal activity and antagonists (327 isolates) were found amongst the dominant culturable bacteria isolated from all six soils. The overall proportion of antagonists was higher in three of the suppressive soils (FR, NL and SE). A higher number of isolates with inhibitory activity against *F. oxysporum* was retrieved from suppressive soils compared to the soils without history of suppression. The highest proportion of antagonists was found in NL soil. Phenotypic characterization of antagonistic bacteria revealed siderophore and protease activity as the most prominent traits amongst the antagonists. Although the majority of the antagonists were identified as *Pseudomonas* spp. (110 of 327) and *Streptomyces* spp. (113 of 327) by FAME and 16S rRNA gene sequencing, high phenotypic and genotypic diversity of antagonists was revealed. The diversity found for antagonists isolated from R2A was considerably higher for all suppressive soils. The composition and diversity of antagonists in each soil was site-specific. Nevertheless, none of the antimicrobial traits of bacteria potentially contributing to soil suppressiveness analyzed in this study could be regarded as specific to a given site.

1. Introduction

Soil-borne pathogenic microorganisms affecting plant health are the main and constant menace to food production worldwide. Over the past few decades, agricultural production has increased and farmers rely on chemical pesticides as a relatively dependable method of protecting plants against soil-borne pathogens (Compant et al., 2005). Nonetheless, the use of agrochemicals causes many negative effects on human´s health and the environments. Consequently, there has been increased restriction on a variety of chemical pesticides. Over the last decades, efforts have been directed towards developing new alternatives to chemical disease control. Many studies have reported on natural activity of some fungi and bacteria against phytopathogens, and this is considered as a very appealing alternative to the use of chemical fungicides (Gerhardson, 2002; Welbaum et al., 2004).

Disease-suppressive soils have been described for several soil-borne plant pathogens, including *Gaeumannomyces graminis* var. *tritici* (Cook and Rovira, 1976), *Fusarium oxysporum* (Alabouvette, 1986), *Plasmodiophora brassicae* (Worku and Gerhardson, 1996), and *Rhizoctonia solani* (Garbeva et al., 2004). Although the abiotic factors of the soil are believed to play a role, there are several evidences that microbial activity contributes to disease suppression (Weller et al., 2002; Mazzola, 2002). Thus, such soils are regarded as sources of natural, effective and valuable antagonists for biological control purpose. Isolates with antagonistic activity towards plant pathogens have been found among the most abundant soil and plant-associated bacteria, such as *Bacillus, Burkholderia, Pseudomonas, Serratia* and *Streptomyces (*Berg et al., 2002, 2006; Feio, 2004*).* Different mechanisms are supposed to contribute to the suppression of soil-borne plant pathogens: parasitism, production of antifungal compounds, competition for ferric iron, nutrients and colonization sites, and inducing systemic resistance in plants against pathogens

(Fravel, 1988; Han et al., 2000; Lugtenberg et al., 2001; Berg et al., 2005; Dahiya, 2005; Kobayashi et al., 2005;).

In this study we assessed the *in vitro* antagonistic potential towards *R. solani* AG3 and *F. oxysporum* in a collection of bacterial isolates obtained from soil samples taken from different geographic locations and with different history of soil suppressiveness. Similar numbers of isolates were retrieved by plating from soils located in France (FR), the Netherlands (NL), Sweden (SE) and the United Kingdom (UK), which have been previously identified as suppressive to *Fusarium oxysporum* f. sp. *lini* (Foln3), *Rhizoctonia solani* AG3, *Plasmodiophora brassicae* and *Gaeumannomyces graminis* respectively. Moreover, isolates retrieved from two other agricultural soils (without known history of phytopathogen suppression) located in Germany were also evaluated. We aimed to determine whether (1) the proportion and diversity of bacteria with antagonistic properties towards *R. solani* and *F. oxysporum* differ between soils with different history of suppressiveness and (2) particular antagonistic attributes can be related to a given site, functioning as a potential indicator of major antagonistic mechanisms that confer suppressiveness to these soils. Total counts of colony forming units (cfu) were determined by plating serial dilutions of soil suspensions onto R2A (total aerobic bacteria), King´s B (selective for *Pseudomonas*) and AGS medium (selective for *Streptomyces*). Similar numbers of bacterial isolates from all sites and media were screened for antagonistic activity by *in vitro* dual culturing. For all *in vitro* antagonists the antibacterial activity, production of extracellular enzymes and siderophores was determined. Representative antagonists were identified by fatty acid methyl ester (FAME) analysis and/or partial 16S rRNA gene sequencing. The genetic diversity of the most frequently isolated *in vitro* antagonists was characterised by BOX-PCR fingerprints.

2. Materials and methods

2.1. Soil and soil sampling

Soil samples were collected from four sites located in France (FR), the Netherlands (NL), Sweden (SE), and the United Kingdom (UK), which were reported previously to be suppressive to phytopathogenic fungi, two agricultural sites in Germany without known history of suppressiveness located in Braunschweig (G-BS) and Berlin (G-BR) were included. All information on the different soils is compiled in Table 3.1.

Sampling of all soils proceeded as follows. From each site, four plots were formed, each of about 15 m by 5 m. Approximately 20 single soil samples from the top layer of the bulk soil (0 – 20 cm) per plot were taken randomly using a sterile auger and mixed to yield one composite sample per plot. Four composite soil samples were collected from each site. Root and surface debris were removed and sieved with a 2 mm sieve.

2.2. Isolation of bacteria and determination of colony forming units

Dilution plating was performed to assess the counts on R2A, King´s B (KMB) and Argine Glucose Salt agar (AGS). Five grams of soil (dry weight) were taken from each composite replicate re-suspended in 45 ml sterile 0.85% NaCl with 15 g glass beads in a sterile Erlenmeyer flask and shaken on a rotating shaker for 15 min at 150 rpm. Samples were serially diluted with sterile saline and plated in duplicates onto R2A (Difco, Detroit, MI, USA), KMB (Merck, Germany) supplemented with chloramphenicol (13 µg ml^{-1}) and ampicillin (100 µg ml^{-1}) (Simon and Ridge, 1974) (KMB+).

Table 3.1. Description of soils from the six sites and the pathogen/disease suppressed

Country/location	% organic matter	pH	Soil type	Cropping history	Pathogen/disease suppressed	Reference
France/ Chateaurenard (FR)	10.5% Organic carbon	8	Silt-clay	Several years cultivated with salad and organic matter fertilization	*F. oxysporum (*Fusarium wilt*)*	Alabouvette, 1986
The Nertherlands/ Wildekamp, Bennekom (NL)	2.5	5.5	Loamy sand	Permanent grass land for more than 20 years	*R. solani* AG3	Garbeva et al., 2004
Sweden/ Uppsala (SE)	1.48% organic carbon	6.9	Clay loam	Cultivated field (recently planted with oat)	*Plasmodiophora brassicae* (Clubroot of cabbage)	Worku and Gerhardson,1996
United Kingdom/Rothamsted "Exhaustion land" (UK)	ND	ND	Silty clay loam	Cultivated field that had serious take-all after switching from continuous barley to wheat in 1992, which subsequently declined	*Gaeumanomyces graminis*	Gutteridge et al., 1996
Germany/ Berlin (G-BR)	2.3	6.4	Sand	Cultivated field (recently planted with Strawberry, oil seed rapes and potato)	Unknown	Berg et al., 2005
Germany/ Braunschweig (G-BS)	1.6	6.0	Weakly loamy sand	Cultivated field (recently planted with Strawberry, oil seed rapes and potato)	Unknown	Berg et al., 2005

ND indicates data not determined

After air-drying of soils for several weeks and incubating them at 60°C overnight serial dilutions were made as described above and plated onto AGS (Herron and Wellington, 1990). Cycloheximide (100 µg ml^{-1}) was added to each medium to inhibit fungal growth. Plates were incubated for seven days at 28℃. Colony forming units (cfu) were counted after seven days.

Per soil, 100 bacterial colonies with visually different colony morphology were picked from dilution plates with 20 to 100 colonies grown on R2A and KMB+ plates. From AGS medium different numbers of *Streptomyces*-like colonies were picked from each soil: UK = 63, NL = 89, F = 111, S = 87, G-BR = 126 and G-BS = 112. All isolates were purified, isolates with morphology similar to *Streptomyces* were stored in trypticase soy broth (TSB) (Becton, Dickinson and Co, Sparks, MD, USA) while other isolates were stored in Luria-Bertani (LB) broth (ROTH, Germany) at -80°C, each medium contained 20% glycerol.

2.3. Screening of isolates for antagonistic activity towards *R. solani* AG3 and *F. oxysporum* f. sp. lini Foln3

Antagonistic activity of 1,788 bacterial isolates against *R. solani* AG3 and *F. oxysporum* f. sp. *lini* Foln3 (basidiomycete and ascomycete, respectively, with a chitin-glucan-containing cell wall) was tested by dual culturing on Waksman agar (WA) containing 5 g of proteose-peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck), 3 g of meat extract (Chemex, München, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco, Detroit, MI, USA), and distilled water (to 1 liter) (pH 6.8). The fungal strain *R. solani* AG3 was obtained from Plant Research International, Wageningen, the Netherlands. *Fusarium oxysporum* f. sp. *lini* Foln3 was received from INRA, Dijon, France. Four 6-mm agar disks containing grown mycelia of either of the two phytopathogenic fungi were placed approximately 3.5 cm (distance) apart

on a WA dish. Four bacterial isolates were streaked between the agar disks. Zones of inhibition around the isolates were measured after 5-7 days of incubation at 20 ℃ (Figure 3.1). All strains were tested in two replicates on different plates and the tests were carried out twice for each isolate.

2.4. Screening of antagonists for anti-bacterial activity against *Ralstonia solanacearum* B3B and *Bacillus subtilis* 1064

Only bacterial isolates which showed antagonistic activity towards *R. solani* AG3 and *F. oxysporum* Foln3 were further screened for antibacterial activity. *Ralstonia solanacearum* B3B (biovar 2; race 3) and *Bacillus subtilis* 1064 were chosen as model organisms for gram negative and gram positive bacteria, respectively. *Ralstonia solanacearum* B3B was obtained from the Federal Biological Research Centre for Agriculture and Forestry (BBA), Kleinmachnow, Germany. *Bacillus subtilis* 1064 was provided by Prof. Christopher Thomas, Birmingham, UK. *R. solanacearum* strain B3B was grown in yeast peptone glucose (YPG) broth for three days at 28^{o}C (YPG medium contains 5 g yeast extract, 5 g bacto peptone, 10 g D-Glucose, 15 g agar and 1 liter distilled water). Approximately 10 ml of the *Ralstonia* suspension and 5 ml of 1% 2, 3, 5,-triphenyl tetrazolium chloride (TZC) (Merck, Darmstadt, Germany) was added to 1 litre sterile YPG containing 1.5% agar when cooled down to about 50^{o}C prior to pouring the medium into Petri dishes. Freshly grown bacterial antagonists were then streaked on the medium and clear haloes without pinkish coloration around the isolates indicating inhibition of *Ralstonia* growth were measured after incubation at 28^{o}C for 72 h (Figure 3.1). For activity against *B. subtilis*, a similar procedure as described for *Ralstonia* was used, except that *B. subtilis* was grown on Trypticase Soy Broth (TSB) (Becton, Dickinson and Co,

Sparks, MD, USA) for 16 hours at 28^{o}C before adding 150 ml of the cell suspensions and 5 ml of 1% TZC to molten TSB containing 1.5% agar.

2.5. Screening of antagonists for production of siderophores and cell-wall degrading enzymes

The ability of the antagonistic bacterial isolates to produce siderophores when grown under Fe^{3+}-limiting conditions was evaluated during a plate assay, according to the method of Schwyn and Neilands (1987). Growth of *Streptomyces* isolates was inhibited on the blue-agar CAS plates. Therefore, the method was slightly modified by overlaying the blue-agar chrome azurol S (CAS) plates with a thin layer (c. 10 ml) of Trypticase Soy Agar (TSA) (Becton, Dickinson and Co, Sparks, MD, USA). Thereafter fresh cultures of *Streptomyces* isolates were streaked on the plates. When the Fe^{3+} is removed from the chrome azurol S complex by high-affinity siderophores, its colour changes from blue to orange. The occurrence of orange halos around colonies was determined after 48 h at 28^{o}C (Figure 3.1).

In order to assess the ability of the antagonists to lyse the cell wall of *R. solani* AG3 and *F. oxysporum* Foln3, they were screened for the production of cell-wall degrading enzymes such as protease, chitinase, glucanase and cellulase. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested in chitin minimal medium, according to the method of Chernin et al. (1995). Clearing zones indicating the enzymatic degradation of chitin were measured after seven days of incubation at 28℃. β -glucanase activity was tested using chromogenic azurine-dyed, cross-linked (AZCL) substrates (Megazym). Formation of blue haloes (Figure 3.1) was recorded until 5 days after incubation (Berg et al., 2002). Cellulase activity was determined using one-tenth volume of TSA containing 0.1% azo-cellulose (Megazym). Degradation of cellulose as indicated by clearing zones was measured after 5 days of

incubation at 28^{o}C. Protease activity (casein degradation) was determined from clearing zones (Figure 3.1) in skim milk agar (50 ml of sterilized skim milk mixed at 55℃ with 50 ml of one-fifth volume of Trypticase S oy Broth and 4% agar) after 5 days of incubation at 28℃ as described by Berg et al. (2002).

2.6. DNA extraction from isolates

A single colony of each isolate was streaked on R2A plate and incubated for 48 h at 28^{o}C; cell mass was re-suspended in 0.85% NaCl and centrifuged at 14,000 xg for 2 min. To obtain crude cell lysates, the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) was used. DNA extraction was performed using the Ultra Clean TM15 DNA Purification Kit (MoBio Laboratories, Carlsbad, CA, USA). DNA yields were checked on UV light (254 nm) after agarose gel electrophoresis and ethidium bromide staining.

2.7. BOX-PCR genomic fingerprints

Whole-genome BOX-PCR fingerprints according to Rademaker et al. (1999) were generated from extracted genomic DNA for most of the antagonistic isolates, especially those that were frequently isolated. BOX-PCR profiles were compared using the software package GelCompar II version 5.6 (Applied Maths, Kortrijk, Belgium). A similarity matrix (Pearson correlation indices) was generated from the profiles obtained and the isolates were clustered by the unweighted pair-group method using arithmetic averages (UPGMA).

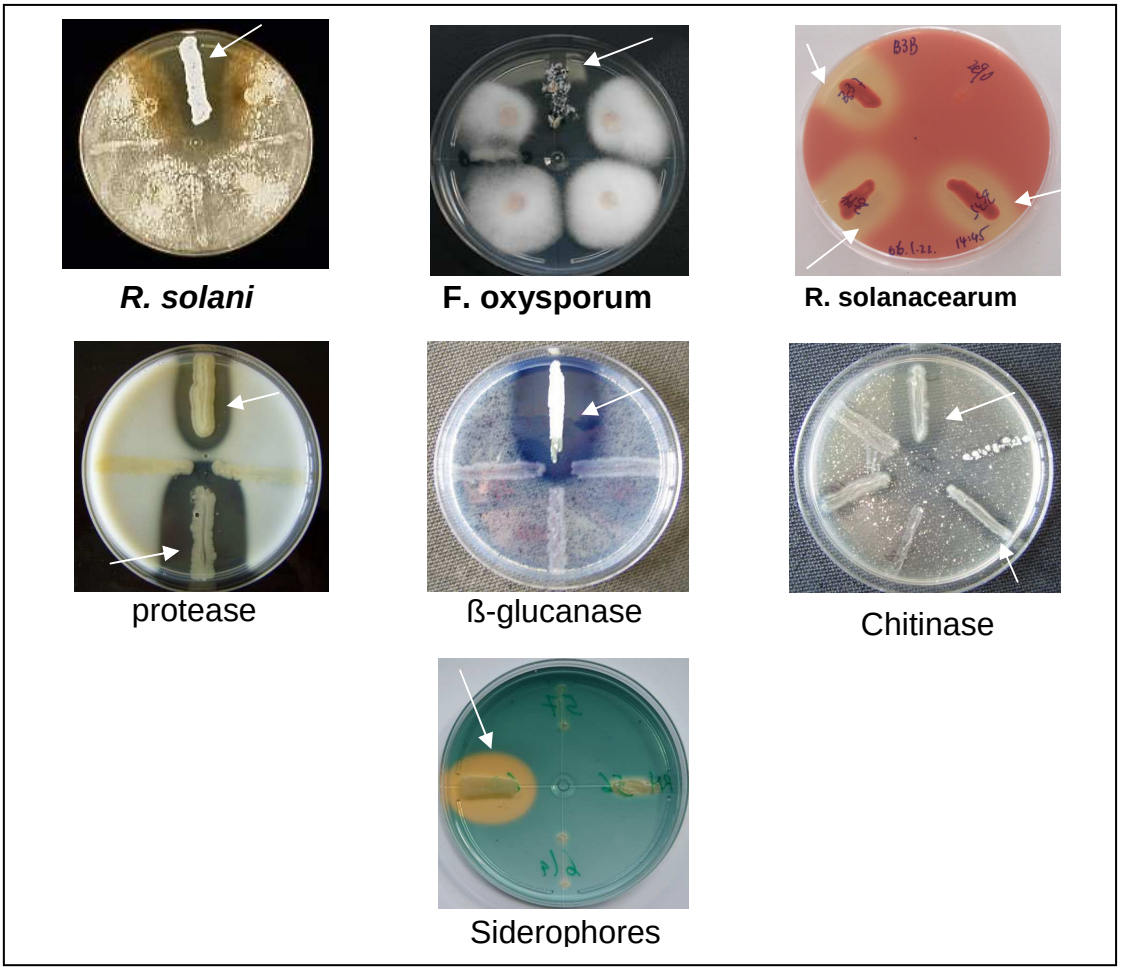

Figure 3.1. Coloured or cleared zones on test plates indicating *in vitro* antagonistic activity towards *R. solani, F. oxysporum*, *R. solanacearum* and *in vitro* production of protease, ß-glucanase, chitinase and sidephores. Inhibition zones or halos from positive strains are indicated with white arrows.

2.8. Identification of bacterial antagonists

A total of 95 antagonists was identified by Fatty Acid Methyl Ester analysis (FAME) of total cellular fatty acids by gas chromatography using the MIDI system (Microbial Identification System, Inc., Newark, USA). In addition, 175 representative antagonists corresponding to different genotypes as revealed by BOX-PCR were submitted to partial sequencing of the 16S rRNA gene. Sequences were aligned with those present in the relevant databases using the nucleotide-nucleotide blastn search tool from NCBI (http://www.ncbi.nih.gov/) and the sequence match tool of the Ribosomal Database Project II (RDP -http://rdp.cme.msu.edu/).

Partial 16S rRNA gene sequences retrieved in this study were deposited in the GenBank database under the accession numbers DQ846813 to DQ846831, DQ847276 to DQ847412 and EF469205 to EF469223.

2.9. Statistical analysis

Bacterial counts (cfu per gram of soil) were logarithm transformed and compared by analysis of variance (ANOVA). Treatment means were separated by Tukey´s test with the SAS statistical package (SAS Institute, Cary, NC, USA). Data were considered to be statistically different at $P< 0.05$.

Principal Components Analysis (PCA) was performed to group antagonists according to their phenotypic characteristics and search for correlations between antimicrobial traits. Data on antagonistic activity assays (towards *R. solani, F. oxysporum, R. solanacearum* and *B. subtilis*), enzymatic activity assays (chitinase, cellulase, β-glucanase and protease) and siderophore production were used to run PCA with the software Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY, USA). As the zones of growth inhibition of phytopathogens on Petri dishes were measured, these

data were used in the PCA in a semi-quantitative manner by generating ranks of strength of antagonism. The sampling sites from where the antagonists were isolated were displayed passively on the ordination diagrams to facilitate the search for possible correlations between antagonistic features and a given soil type. PCA was also carried out to compare the different soils with respect to their composition and diversity of culturable antagonistic bacteria retrieved from R2A. Due to the high diversity of antagonistic bacteria retrieved from these soils, analysis of composition and diversity of antagonists was done at the genus level. An inventory was created listing the genera (as identified by 16S rRNA gene sequencing or by FAME) of antagonistic bacteria observed and their respective abundances (i.e., number of antagonists belonging to each genus) for each of the soils studied. Euclidean distances between the soil samples were calculated based on presence/absence and relative abundance data of each genus in each sample. The diversity of antagonistic bacteria retrieved in each soil was calculated using the Shannon measure of diversity (H'), determined as $H' = -\sum pi \log pi$, where *pi* represents the relative abundance of genus *i* within the community of culturable bacterial antagonists. PCA ordination biplots of sampling sites and bacterial genera were generated to display differences in composition and diversity of antagonistic isolates in each soil by graphic means.

3. Results

3.1. Plate counts

To estimate the number of culturable aerobic bacteria in the different soils plate counts were determined on R2A. The total counts on R2A ranged from 5.7 x 10^5 to 10^7 cfu per gram of soil (Figure 3.2). Significantly lower cfu counts on R2A were

obtained for NL soil (5.7 x 10^5) compared to all other soils. Counts on KMB+ were in the range of 2.6 10^3 to 7 x 10^5. On KMB+ by far the lowest counts were recovered for G-BR soil. The highest cfu counts on the selective medium for *Pseudomonas* (KMB+) were found for G-BS, SE and FR soils. On the *Streptomyces* selective medium (AGS) again the lowest counts were observed for the NL soil while the highest cfu counts were found for FR soil and both soils with no history of suppressiveness (G-BR, G-BS).

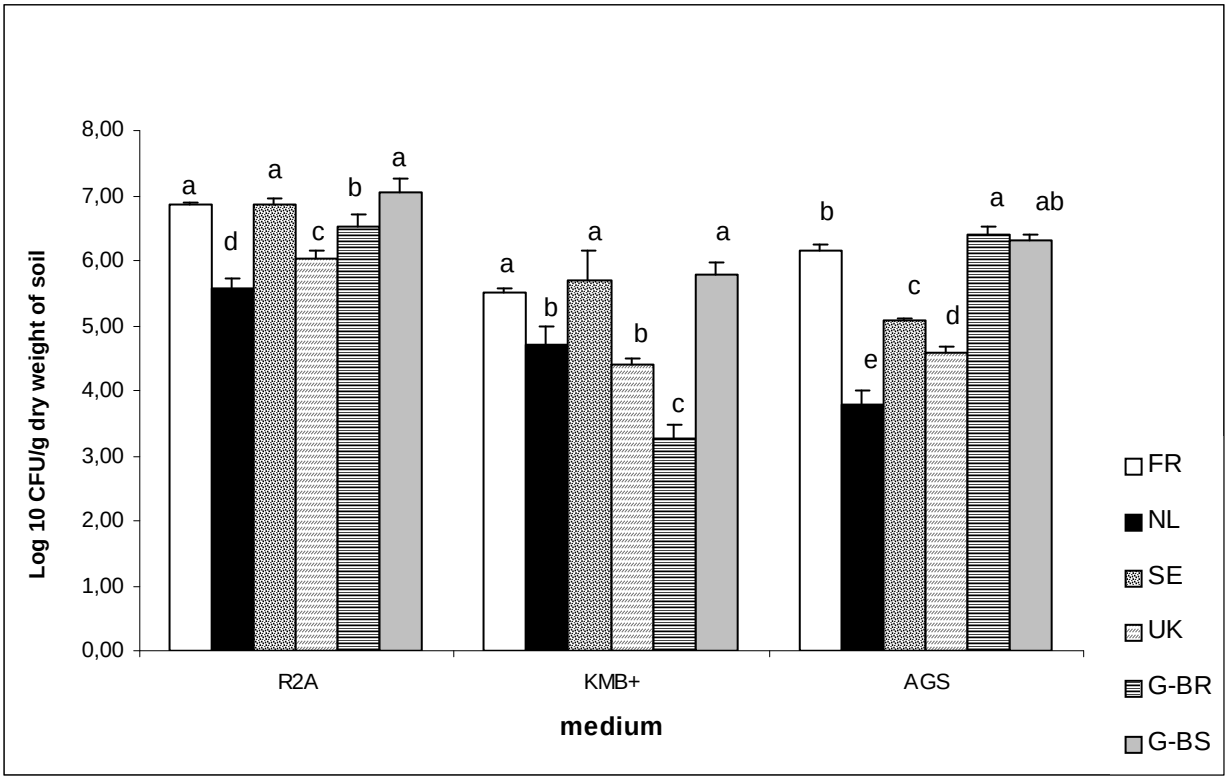

Figure 3.2. Culturable bacterial population in soil with and without history of disease suppression obtained on R2A, KMB+ and AGS media. Values obtained on the same medium with the same letter are not significantly different (P< 0.05) according to the Tukey´s test.

3.2. Determination of the in vitro antagonistic activity towards *R. solani* and *F. oxysporum*

A total of 1,788 bacteria isolated from R2A, KMB+ and AGS was screened for antagonistic activity against *R. solani* AG3 and *F. oxysporum* Foln3 in an *in vitro* dual culture assay. Approximately 18.3% of the isolates (327 of 1,788) displayed antagonistic activity towards one or both of the phytopathogenic fungi. Inhibition was clearly discerned by limited growth or the complete absence of fungal mycelium in the inhibition zones surrounding the streak of the isolates tested (Figure 3.1). Isolates with *in vitro* antagonistic activity were found amongst the dominant culturable bacteria retrieved from all six soils. Overall the total proportion of antagonistic bacteria isolated from three different media was clearly higher for three of the four suppressive soils: NL (28%), FR (23%) and SE (22%) (Table 3.2), compared to G-BR (11%) and G-BS (14%) and the UK soil (12%). The proportion of antagonistic bacteria found in the R2A, KMB+ and AGS collections was remarkably different for each site (Table 3.2). The number of isolates from R2A, which displayed antagonistic activity is a measure of the proportion of *in vitro* antagonists amongst the total aerobic bacteria in each of the soils. Although the lowest cfu counts on R2A and AGS were determined for NL soil, interestingly, the highest proportion of *in vitro* antagonists towards *R. solani* and *F. oxysporum* isolated from all media was found for this soil (28% of 289; R2A: 25%; KMB+: 25%; AGS: 36%). The proportion of antagonists in the R2A collection from FR soil was comparable with NL soil, however, a much higher proportion of KMB+ isolates (39%) and a lower number of AGS isolates (6.3%) was found for FR soil (Table 3.2). Approximately 20% of the R2A isolates from UK soil showed antagonistic activity. However, the proportion of KMB+ isolates (6%) and AGS (8%) isolates was rather low. In contrast, the lowest number

of the R2A isolates with antagonistic activity was obtained from SE soil (10/100) while 40% of isolates from KMB+ and 16% from AGS displayed antagonistic activity.

Although natural suppression of *R. solani* AG3 was documented only for NL soil, bacteria with the ability to suppress *R. solani* AG3 were isolated from all soils. The highest proportion was indeed observed for the NL soil (18% of 289) followed by SE soil (15% of 287). However, there were no clear differences between soils with and without documentation of disease suppression (Table 3.2). Likewise, *F. oxysporum* antagonists were isolated from all soils and not only from soils with known suppressiveness towards this pathogen. Despite similar numbers of bacteria tested from R2A and KMB+, proportion of antagonists found in each medium was different for all soils (Table 3.2). The proportion of *F. oxysporum* antagonists was clearly higher in all suppressive soils. Even though, FR soil was described as suppressive towards *F. oxysporum* the highest proportion of antagonists was again found for the NL soil (20% of 289) followed by FR soil (17% of 311), SE soil (13% of 287), UK soil (9% of 263).

3.3. Population of antagonists with antibacterial activity

All 327 antagonists (NL=82, FR=71, SE=64, G-BS=44, G-BR=35, UK=31), that exhibited antifungal activity against at least one of the two pathogenic fungi, were further screened *in vitro* for antibacterial activity as indication of antibiotic production. *Ralstonia solanacearum* strains B3B (a causal agent of bacterial wilt) and *Bacillus subtilis* strain 1064 were selected as model organisms for gram-negative and gram-positive bacteria, respectively.

Table 3.2. Total number of isolates with anti-fungal activity against R. solani and F. oxysporum isolated on R2A, KMB+ and AGS media from the six soils

Site	Medium	Total isolates	Total no. of antagonists	Total no. of *R. solani* AG3 antagonists	Total no. of *F. oxysporum* antagonists
FR	R2A	100	25	15	15
	KMB+	100	39	12	35
	AGS	111	7 (6.3%)	5 (4.5%)	2 (1.8%)
NL	R2A	100	25	10	21
	KMB+	100	25	13	16
	AGS	89	32 (36%)	30 (34%)	22 (25%)
SE	R2A	100	10	6	6
	KMB+	100	40	28	25
	AGS	87	14 (16%)	9 (10%)	7 (8%)
UK	R2A	100	20	11	16
	KMB+	100	6	0	6
	AGS	63	5 (8%)	4 (6%)	1 (1,5%)
G-BR	R2A	100	15	13	10
	KMB+	100	3	3	1
	AGS	126	17 (14%)	15 (12%)	6 (5%)
G-BS	R2A	100	11	8	7
	KMB+	100	15	15	4
	AGS	112	18 (16%)	15 (13%)	6 (5%)
Overall	R2A	600	106	63	75
	KMB+	600	128	71	87
	AGS	588	93	78	44

Isolates with the ability to suppress the growth of the two selected model bacteria strains could be easily detected using tetrazolium hydrochloride containing YPGA or LB media with *R. solanacearum* or *B. subtilis* (Figure 3.1). Isolates inhibiting the growth of *R. solanacearum* and/or *B. subtilis* were found in all soils. With exception of UK soil, a higher proportion of the antagonists from suppressive soils (44 of 82 for NL, 39 of 64 for SE, and 25 of 71 for FR) inhibited the growth of *R. solanacearum* in comparison to the antagonists from the soils without history of suppression (11 of 44 for G-BS and 9 of 35 for G-BR) (Table 3.3). Antagonistic activity against *R. solanacearum* was found in more than half of the antagonists from SE and NL soils, and was frequently observed amongst isolates obtained on KMB+ from SE (56%), NL (30%) and FR (35%).

NL was again the soil where the highest frequency of antagonists active against *Bacillus subtilis* (32%) was found (Table 3.3). A similar but slightly lower frequency was observed also amongst the antagonists from G-BR (31%). Antibacterial activity against *Bacillus subtilis* was frequently observed amongst antagonists obtained on AGS (22%) and KMB+ (20%) media from NL and SE soils, respectively. Overall, with exception of UK and G-BR, a larger number of antagonists displayed inhibitory activity towards the gram-negative bacterium *R. solanacearum* than towards the gram-positive bacterium *B. subtilis* (Table 3.3).

Broad-spectrum *in vitro* activity against the two fungi and two bacteria used as model organisms in this study was displayed by 16 of the total antagonists (16/387: NL-6, SE-5, FR-2, G-BR-2, G-BS-1).

3.4. Frequencies of siderophore and lytic enzyme producing antagonists

To elucidate further potential mechanisms of fungal and bacterial inhibition, the collection of *in vitro* antagonists towards *R. solani* and *F. oxysporum* was

screened for production of siderophores and lytic enzymes (Figure 3.1 and Table 3.3). A high proportion of the antagonists isolated from all six soils produced siderophores (272/327) but this trait was particularly abundant in FR soil (68/71) (Table 3.3). High siderophore production as indicated by a wide zone of orange colour on CAS plates (Figure 3.1) was frequently observed among *Pseudomonas* antagonists obtained from KMB+ for all soils. Protease activity was also displayed by the majority of the antagonists (75%).

Except for FR soil where the highest proportion of antagonists (46%) showed chitinase activity, a higher frequency of chitinase producers was found in soils without documentation of suppression than in suppressive soils (Table 3.3). A remarkably low proportion of antagonists from NL soil (5%) displayed chitinase activity.

3.5. Identification of antagonists by fatty acid methyl ester analysis or 16S rRNA gene sequencing

A total of 95 non-Streptomyces antagonists was identified by FAME. All strains which had a FAME index below 0.4 and all Streptomyces isolates were first screened by BOX-PCR from genomic DNA and subsequently representatives of each BOX type (175 strains) were identified by partial 16S rRNA gene sequencing. Table 3.4 gives an overview of the bacterial genera to which the antagonists isolated from the six different soils could be assigned. More detailed information is given in Appendix 1. The highest number of species (37) was found in FR soil (Table 3.4). Most frequently isolated antagonists from FR soil were identified as *Streptomyces* (18) and *Stenotrophomonas maltophilia* (12).

Table 3.3. Proportion (%) of antagonists with antibacterial activity and production of lytic enzyme and siderophores

Site/total antagonists	Antibacterial activity		Lytic enzymes activity				Siderophore
	R. solanacearum	*B. subtilis*	Protease	Glucanase	Cellulase	Chitinase	
FR (n = 71)	35	10	61	45	15	46	94
NL (n = 82)	54	33	74	29	15	5	77
SE (n = 64)	61	23	94	22	23	23	89
UK (n = 31)	19	19	77	23	23	39	90
G-BR (n = 35)	26	31	69	77	66	46	63
G-BS (n = 44)	25	16	75	39	41	41	80

Table 3.4. Numbers of bacterial genera (OTU) with antagonistic activity retrieved from the different soils

Operational taxonomic unit (OTU)	FR	NL	SE	UK	G-BR	G-BS
Alphaproteobacteria	9 (5)					
Betaproteobacteria	4 (1)	2 (1)	1	4		
Gammaproteobacteria						
Dyella		(4)				
Enterobacter	3 (2)					
Yersinia	1					
Lysobacter	1					
Pseudomonas fluorescens	4 (3)	16 (15)	25 (21)	(4)	(2)	(7)
Pseudomonas putida	4 (1)	13	5		(1)	
Other *Pseudomonas* spp.	2	4 (3)	8 (1)	6 (5)		(9)
Stenotrophomonas maltophilia	12 (4)		4 (3)	2 (1)		(3)
Flavobacteria (Chryseobacterium)				1		
Bacilli						
Bacillus	3	6 (2)			(10)	(5)
Brevibacillus	1				(1)	
Paenibacillus				1	(3)	
Staphylococcus	1					
Kurthia	1					
Actinobacteria						
Arthrobacter	(1)	2		4(2)		
Brevibacterium			(1)			
Kytococcus		1				
Microbacterium	(4)					
Micrococcus	1		(1)	1		
Nocardiopsis			(1)			
Rhodococcus			(1)			
Streptomyces caviscabies	(2)	(1)	(10)	(4)	(3)	(2)
Other *Streptomyces* spp.	(16)	(31)	(3)	(3)	(12)	(17)
Unidentified						
Streptomyces-like strains	1	1	3	1	2	1
Others		1	1		1	
Total number of species	37	21	20	15	21	25
Total number of antagonists	71	82	64	31	35	44

Numbers in parentheses are numbers of isolates that could be assigned to a given genus based on partial 16S rRNA gene sequencing of representative BOX-PCR genotypic groups.

Antagonists belonging to the Alphaproteobacteria (*Bosea, Ochrobactrum, Rhizobium, Sinorhizobium, Xanthobacter*) were only isolated from FR soil (Table 3.4 and 3.5). Other genera which were only found in FR soil are *Achromobacter* (1), *Alcaligenes* (1), *Burkholderia* (1), *Enterobacter* (3), *Yersinia* (1), *Lysobacter* (1), *Staphylococcus* (1) *Kurthia* (1) and *Microbacterium* (4) (Table 4). Although the highest number of antagonists was isolated from NL soil only 21 different species were identified. Most remarkable was the high number of antagonists assigned to *Streptomyces* (32) with *S. pulveraceus* (9), *S. purpureus* (7) and *S. sporoc*livatus (12) and to *Pseudomonas* (33) with *P. fluorescens* (16) and *P. putida* (13) as major constituents. The highest proportion of antagonists from Swedish soils belonged to the genus *Pseudomonas* (38/64) and 25 of the *Pseudomonas* strains were identified as *P. fluorescens*. *Streptomyces* isolates were the second biggest group of antagonists from SE soil with most isolates identified as *S. caviscabies*.

3.6. Composition of R2A antagonists retrieved from each soil

In order to display the assemblage of antagonists retrieved from each soil by graphic means, we run a PCA to ordinate the different sites according to data on presence/absence and relative abundances of the antagonistic genera isolated on R2A in each location (Figure 3.3). Analysis was performed at the genus level to facilitate data visualization and avoid identification biases at the species level. Antagonists isolated on KMB+ (*Pseudomonas*) and AGS (*Streptomyces*) were excluded from the analysis because their dominance in numbers would obviously mask potential differences in composition among the sites. Nevertheless, both *Pseudomonas* and *Streptomyces* antagonists were frequently isolated from R2A. Overall they corresponded, together with *Bacillus*, to the most dominant antagonistic genera retrieved from R2A plates (Figure 3.3). Interestingly, suppressive soils (FR,

NL, SE and UK) displayed both higher richness and diversity of antagonists than soils with no history of suppression (Figure 3.3). High diversity of antagonistic bacteria was observed especially in French soil, where 14 different antagonistic genera were isolated on R2A. Among them, 7 were retrieved exclusively from this site (Figure 3.3). The Berlin site was characterized by the exclusive isolation, on R2A, of antagonists (n=14) belonging to the gram-positive genera *Bacillus* (n=10), *Paenibacillus* (n=3) and *Brevibacillus* (n=1). Although antagonists of these genera (especially *Bacillus*) have also been isolated from other sites, this trend marks the differentiation between the Berlin soil and the soils with history of suppressiveness in the ordination space, whose diversity of antagonistic genera is higher (Fig 3.3). In all, the data set on antagonists isolated from R2A indicates that the composition of culturable, antagonistic heterotrophic bacteria in the soils studied differs.

3.7. BOX-PCR fingerprints of most frequently isolated bacterial genera

To assess the diversity of antagonistic isolates from the six sites assigned to the same species based on FAME or 16S rRNA gene sequencing, BOX-PCR fingerprints were generated from genomic DNA. One intriguing question was whether antagonistic strains with high genomic similarity could be isolated from soil taken in different countries. A total of 58 isolates originating from the six soils were identified as *Pseudomonas fluorescens* with most isolates from the SE (n=25) and NL (n=17) soils. BOX-PCR fingerprints of all the *P. fluorescens* isolates revealed a high genotypic diversity amongst the antagonists (Figure 3.4a). At a cut-off of about 70% twelve clusters and eleven single isolates could be identified. Nine of the clusters contained isolates from different countries.

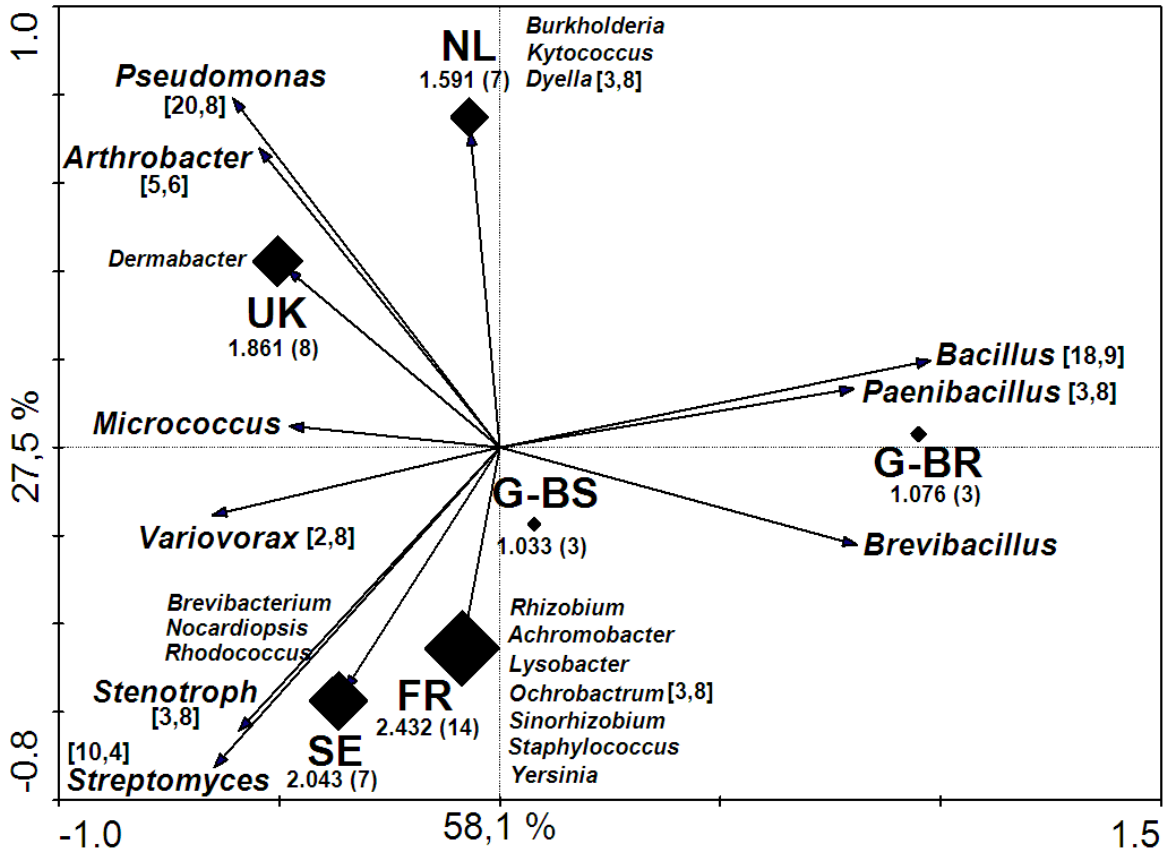

Figure 3.3. PCA ordination biplot of sampling sites (solid diamonds) and genera of culturable antagonistic bacteria (arrows) isolated from R2A. Composition of bacterial antagonists (at the genus level) isolated from each soil was used to calculate measures of dissimilarity between the soil types (G-BS, Braunschweig, Germany; G-BR, Berlin, Germany; FR, France; NL, the Netherlands, SE, Sweden; UK, United Kingdom). Sampling sites are plotted in the ordination space as determined by their Euclidean distances. Diversity indices (at the genus level) were estimated for each soil and are displayed below the sampling sites' abbreviations. The size of each solid diamond corresponds to the diversity index estimated for the respective soil. Number of antagonistic genera found in each soil is given in brackets. Larger font size was used to indicate genera found in more than one sampling site. Genera which were exclusive to a given site are indicated with smaller font size. The relative abundances (%) of dominant genera isolated from R2A (number of antagonists assigned to a given genus divided by the total number of antagonists) are shown next to their names. In case the direction pointed by an arrow (i.e., genus) converges to the position of a given soil type, this indicates that antagonists belonging to that genus were predominantly isolated from the corresponding site.

The largest clusters contained eight isolates originating from two sites (NL and SE) while the second to the largest cluster is comprised of six isolates from SE.

Antagonists belonging to *Streptomyces caviscabies* (n=22) were isolated from all soils but only from AGS medium. The highest number of *S. caviscabies* originated from SE soil (10/23). The BOX patterns showed a high degree of diversity (Figure 4b). At a similarity level of 70% four clusters were formed of which one was composed of strains from two countries (UK and NL).

Pseudomonas putida (n=23) and *Stenotrophomas maltophilia* (n=21) were other frequently encountered antagonistic species isolated in four soils. While the former was isolated in FR, SE, NL, and G-BR, the later was found in FR, SE, UK, G-BS. For each bacteria species, the isolates were highly diversed and at 70% level of similarity, no cluster was formed among isolates from the different sites (Figure 4c and 4d). Chitinase activity was a common phenotypic trait of all the isolates identified as S. *maltophilia,* except three isolates, which originated from the G-BS soil. Similarly*,* siderophores and protein production was found in all S. *maltophilia* antagonists except one. Also 18 of 21 S. *maltophilia* antagonists showed antagonistic activity against *F. oxysporum*.

3.8. Grouping of antagonists according to their antimicrobial attributes

Figure 3.5 is an ordination biplot of antagonists (n=327) and potential antimicrobial traits as determined by principal components analysis (PCA). As all antagonists were included in this analysis, the two targeted genera *Streptomyces* and *Pseudomonas*, isolated on selective media AGS and KMB+, respectively, correspond to the majority of isolates represented in the ordination space.

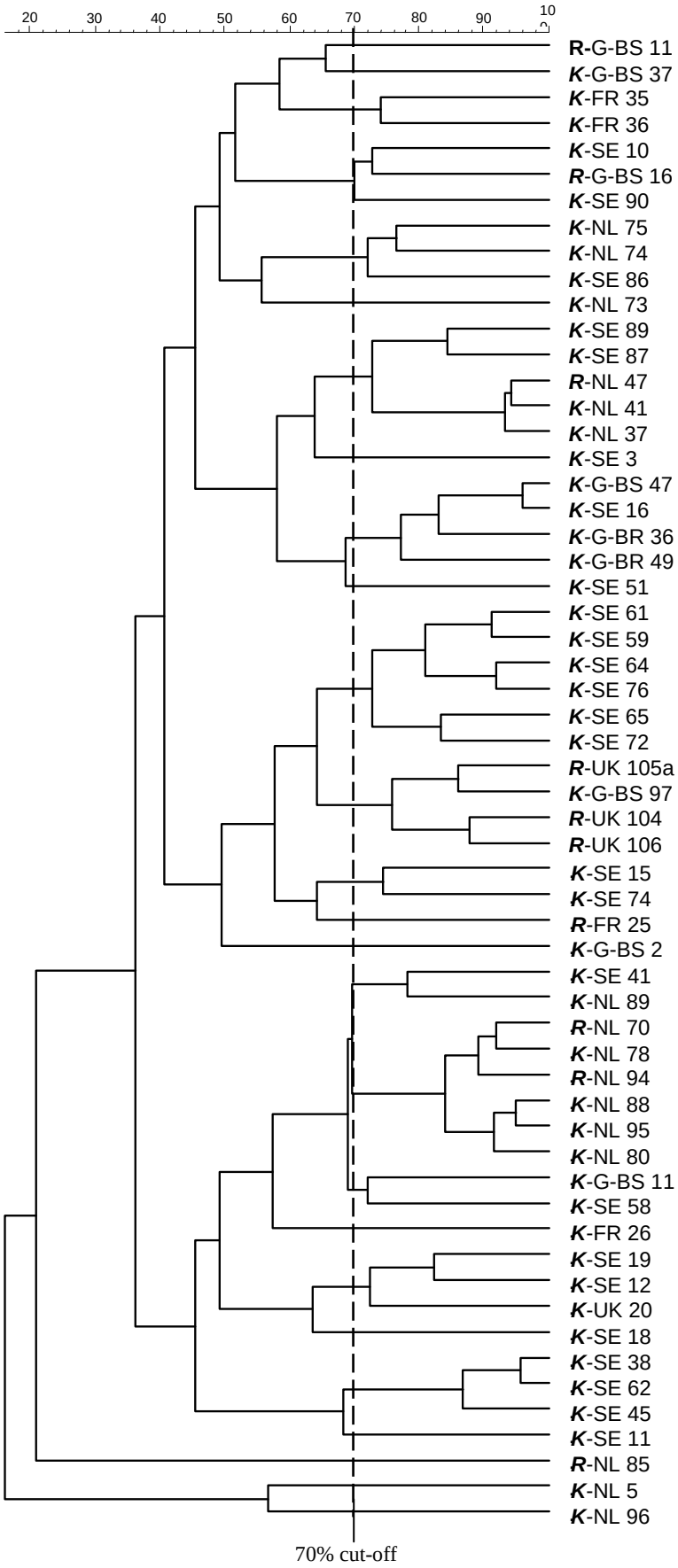

Figure 3.4a: Genotypic comparison among isolates identified as *Pseudomonas fluorescens* Dendrogram generated by cluster analysis of the band pattern obtained from BOX-PCR fingerprints. First italicized letter in bold indicates the medium of isolation (R, R2A; and K, KMB+) followed by the isolate origin and identification number.

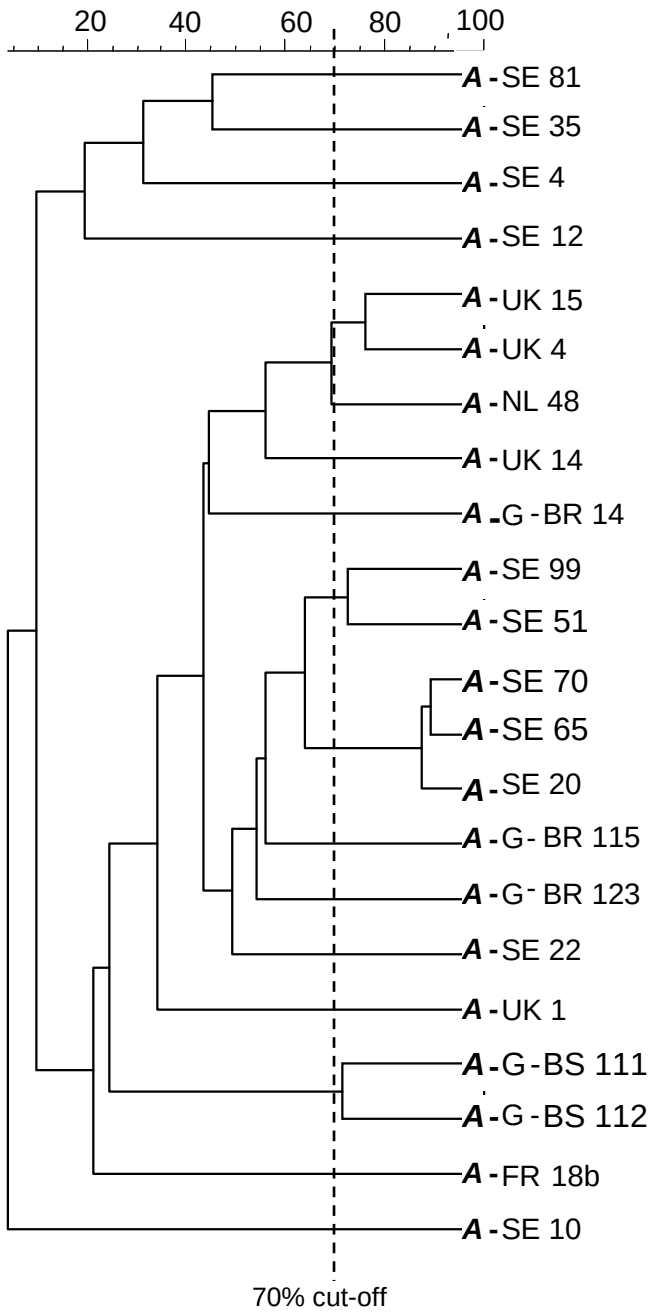

Figure 3.4b. Genotypic comparison among isolates identified as *Streptomyces caviscabies*. Dendrogram generated by cluster analysis of the band pattern obtained from BOX-PCR fingerprints. First italicized letter in bold indicates the medium of isolation (A; AGS) followed by the isolate origin and identification number.

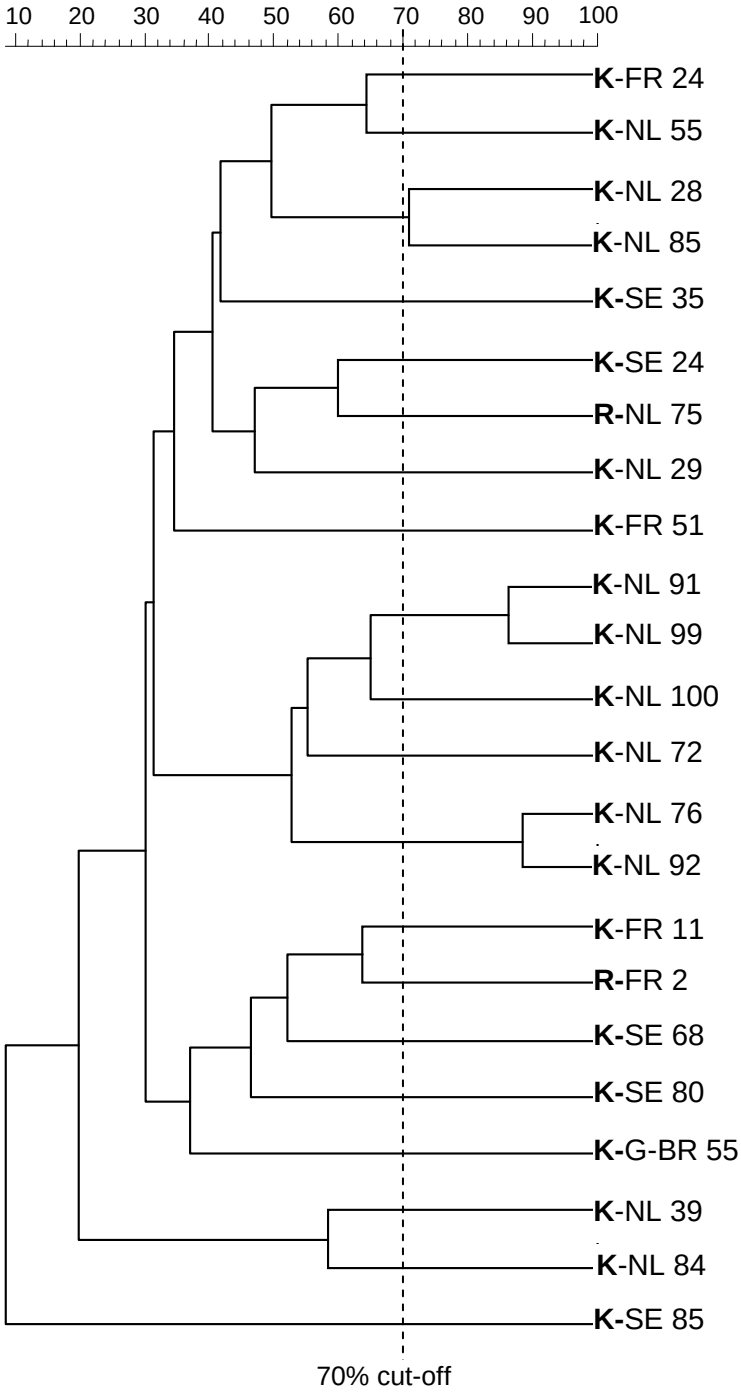

Figure 3.4c: Genotypic comparison among isolates identified as *Pseudomonas putida*. Dendrogram generated by cluster analysis of the band pattern obtained from BOX-PCR fingerprints. First italicized letter in bold indicates the medium of isolation (R, R2A; and K, KMB+) followed by the isolate origin and identification number.

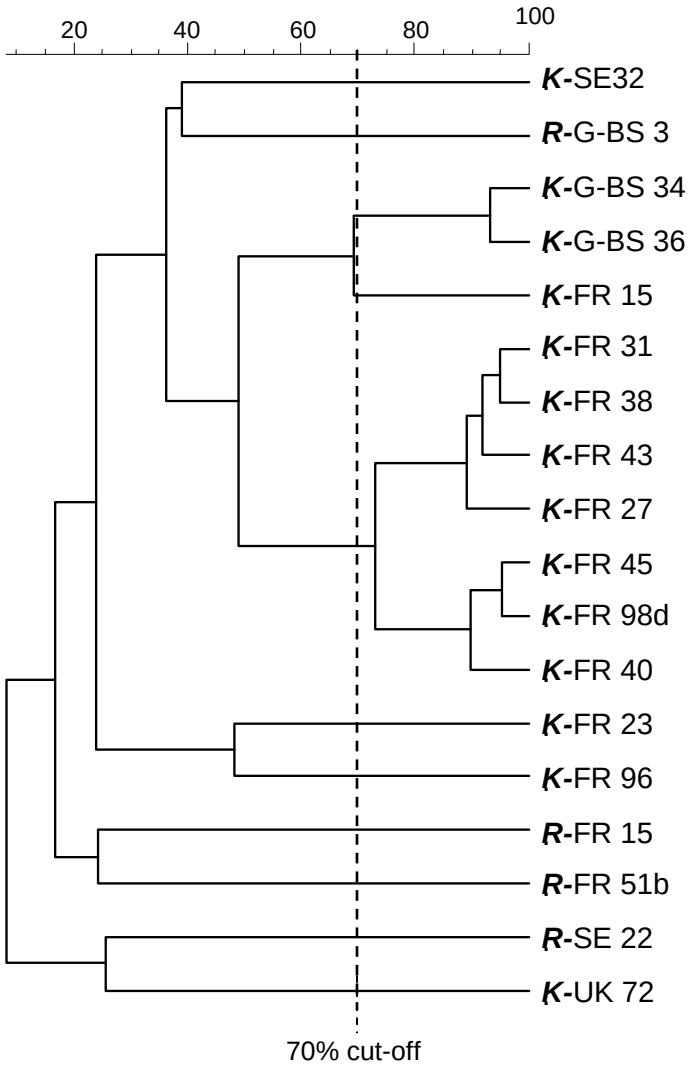

Figure 3.4d: Genotypic comparison among isolates identified as *Stenotrophomonas maltophilia*. Dendrogram generated by cluster analysis of the band pattern obtained from BOX-PCR fingerprints. First italicized letter in bold indicates the medium of isolation (R, R2A; K, KMB+) followed by the isolate origin and identification number.

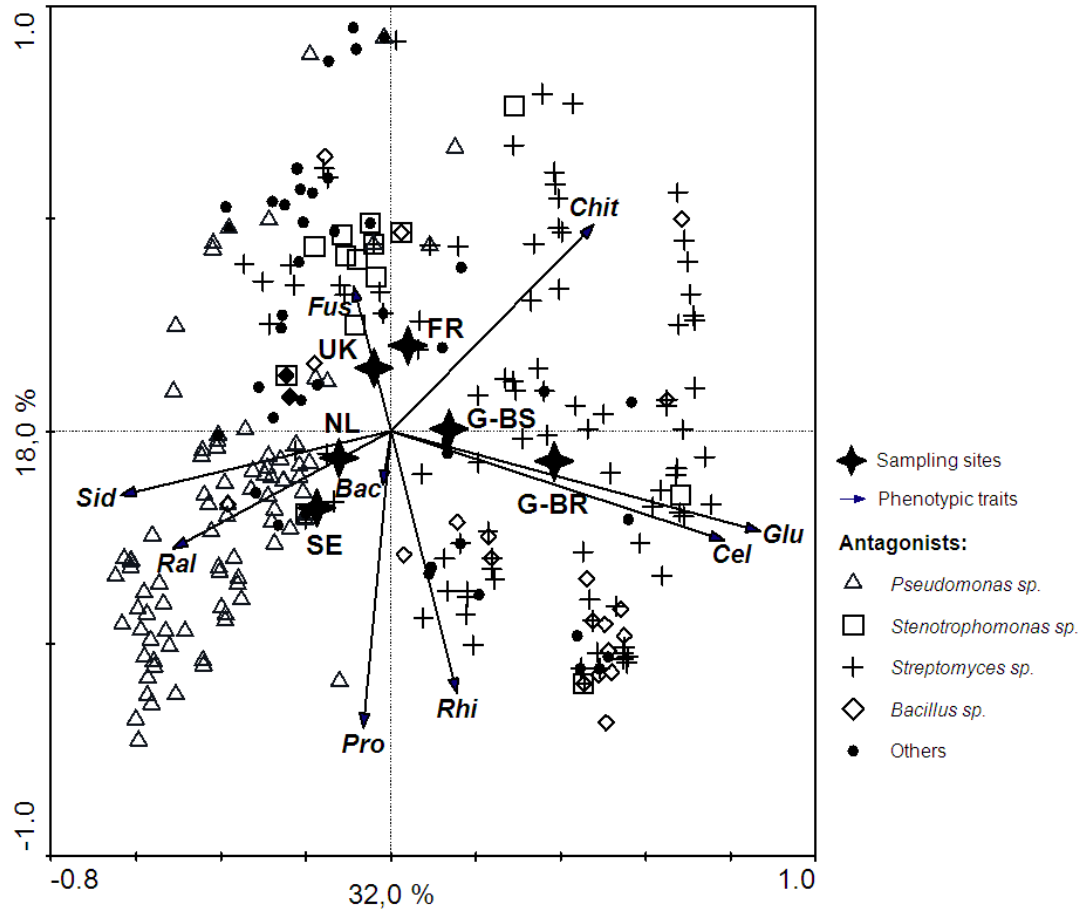

Figure 3.5. PCA ordination biplot of antagonists (symbols) and their antimicrobial properties (arrows). A phenotypic profiling was used to generate measures of dissimilarity among the antagonists. Ranks of intensity (semi-quantitative data) were created to register the data on *in vitro* antagonistic activity towards *Rhizoctonia solani* (*Rhi*), *Fusarium oxysporum* (*Fus*), *Ralstonia solanacearum* (*Ral*) and *Bacillus subtilis* (*Bac*). Binary data (1 or 0) were used to register results for enzymatic activity assays (*Cel*, cellulase; *Chit*, chitinase; *Glu*, glucanase; *Pro*, protease) and siderophore production (*Sid*). Antagonists with similar phenotypic profiles are, thus, plotted near to each other in the ordination space. Most representative genera found among the antagonists were assigned specific symbols (see legend). Lengths of arrows reflect, approximately, the extent to which a given antimicrobial trait contributes to the formation of the ordination space. Angles between arrows represent correlations (positive, negative or neutral) between antimicrobial attributes. Sampling sites were displayed passively (i.e., data on the origin of the isolates do not influence their ordination) in the ordination diagram as determined by their centroid positions.

A clear dichotomy between the antimicrobial properties of *Pseudomonas* and *Streptomyces* antagonists can be depicted from the ordination diagram: while the former group was typically characterized by siderophore production and high potential to antagonize *Ralstonia solanacearum*, the latter was frequently observed to display cellulase and glucanase activity (Figure 3.5). Moreover, chitinase activity was also found to be a typical trait of *Streptomyces*. The above mentioned antimicrobial traits were found, thus, to influence the variation in the data set to a large extent, determining the distribution of antagonists along the horizontal axis - which accounts for 32% of the cumulative data variation - of the ordination diagram (Figure 3.5). The grouping of antagonists along this axis basically sets the differentiation between *Pseudomonas* spp. and *Streptomyces* spp. in the ordination space. The positive correlation between antagonistic activity towards *R. solanacearum* and siderophore production is evidenced in Figure 3.5. In fact, among the 135 isolates active against *R. solanacearum*, 124 were able to produce siderophores under the conditions set in this study. From this group, 91 antagonists were identified as *Pseudomonas* and this trend could also be very well displayed in the ordination biplot generated by PCA (Figure 3.5). The same occurs for the positive correlation between glucanase and cellulase activity (Figure 3.5). In this case, 61% of the strains with glucanase activity showed cellulase activity. The Glu+Cel+ phenotypes (n=74) were mainly observed among antagonists belonging to the genera *Streptomyces* (53 of 74) and *Bacillus* (16 of 74) (Figure 3.5). In all, antagonists with diverse antimicrobial characteristics were retrieved from all soils studied. Because of this, no indications for a positive correlation between a specific phenotypic trait and a given soil (sampling site) was found. This is demonstrated in Figure 3.5, where the centroid positions of each location tend to be situated next to the diagram's origin, indicating absence of

correlation between these sites and any of the antimicrobial properties surveyed in this study. The exception may be the Berlin soil, where gram-positive antagonists were dominant and, as a consequence, cellulase and glucanase activity was most often observed.

4. Discussion

In this study, we used a cultivation-based approach to characterize the *in vitro* antagonistic potential towards *R. solani* and *F. oxysporum* in four soils with history of disease suppression from different geographic locations in Europe. In addition, two agricultural soils without known history of disease suppression were included. To determine the abundance and composition of bacterial antagonists towards *F. oxysporum* and *R. solani,* we isolated the same number of dominant culturable bacteria from the different soils by plating onto R2A. In addition, plating onto media selective for *Pseudomonas* (KMB+) and *Streptomyces* (AGS) was done because these bacterial genera have been previously reported as *in vitro* antagonists (Abd-Allah, 2001; Berg et al., 2002; Michaud et al., 2002; Garbeva et al., 2004).

A comparison between the bacterial antagonists in the six soils was possible because similar numbers of isolates from all soils obtained on the different media were screened by dual tests for *in vitr*o antagonistic activity towards *R. solani* and *F. oxysporum.* Most of the antagonists were identified; their taxonomic composition and diversity in each soil was determined.

Remarkable differences in the cfu counts per gram of soil were observed (up to two orders of magnitude), with NL soil displaying the lowest cfu counts on R2A and AGS. A considerable proportion of the dominant culturable bacteria obtained by plating on R2A displayed *in vitro* antagonistic activity ranging from 10-25%. Bacteria with *in vitro*

antagonistic potential towards *R. solani* and *F. oxysporum* could be isolated from all soils independent from a previous report of fungal disease suppressiveness. This finding supports earlier observations that, virtually, all natural and agricultural soils studied so far possessed some ability to suppress the activity of soil-borne plant pathogens due to the presence and activity of soil microorganisms, a phenomenon referred to as general suppression or general antagonism (Hornby, 1983; Cook and Baker, 1983; Weller et al., 2002; Mazzola, 2004). However, a significantly higher proportion of antagonists was recovered from three of the four suppressive soils compared to soils without history of suppression. In particular, the proportion of bacterial isolates that inhibited *F. oxysporum* was significantly higher for these suppressive soils. The highest proportion of bacterial isolates active against *Rhizoctonia solani* was found in NL soil compared to other soils, which correlates with the natural suppressiveness of this soil towards *R. solani* (Garbeva et al., 2004, 2006). Our results also revealed a slightly higher proportion of bacteria with *in vitro* antagonistic activity against *F. oxysporum* in NL soil that was even higher than that obtained in FR (a soil with documentation of *F. oxysporum* suppressiveness). In addition, we found evidence that SE and NL soil might possess natural potential to suppress *R. solanacearum,* as revealed by more than 50% of antagonists isolated from SE and NL soils displaying inhibitory activity against the bacterial pathogen.

The majority of *in vitro* antagonists were identified as *Pseudomonas* spp. (110/327) and *Streptomyces* spp. (113/327). Obviously, this outcome reflects the strategy employed in this study, where selective nutrient media (KMB+ and AGS, respectively) were used to target these two bacterial genera. Nevertheless, both *Pseudomonas* and *Streptomyces* were also the most frequently isolated antagonists from R2A. *P. fluorescens* (58/110) and *P. putida* (23/110) were the most frequently isolated *Pseudomonas* spp. in six and four soils, respectively. Antagonistic activity of

both species against many soil-borne plant pathogens has also been reported by several other authors (Berg et al., 2002; de Boer et al., 2003; Costa et al., 2006a). Except for the G-BR soil, antagonists identified as *Pseudomonas* spp. were isolated from R2A from all soils, indicating that in these soils *Pseudomonas* spp. with antagonistic activity belonged to the dominant culturable fraction of heterotrophic bacteria. The larger numbers of *Pseudomonas* antagonists retrieved from R2A were observed in NL and UK soils (9/25 and 8/10). Interestingly, R2A isolates from UK soil which were assigned to *Pseudomonas jessenii* were not recovered from KMB+. Berg et al. (2006) reported that the majority (75%) of *Verticillium* antagonists isolated on R2A from oilseed or strawberry rhizosphere or bulk soil from three different sites in Germany were identified as *Pseudomonas* spp. In contrast to the study by Berg et al. (2006) *Pseudomonas* isolates with antagonistic activity were less frequently obtained from R2A (20%) in this study, where analyses were performed exclusively for bulk soils. This trend suggests that an increased abundance of *Pseudomonas* populations with antagonistic activity in the vicinity of plant roots might occur.

Streptomyces spp. with antagonistic activity were also not only isolated from the *Streptomyces* selective medium, AGS, but also from R2A (except for G-BR soil), underlying that they belong to the dominant culturable fraction in bulk soil. From all six soils *Streptomyces* isolates with antagonistic activity were obtained for which the 16S rRNA gene sequence showed the highest similarity to *S. caviscabies.* Eleven of the 22 *S. caviscabies* isolates originated from SE soil. Although our data indicate that *S. caviscabies* populations carrying antimicrobial properties might be abundant in soils with documentation of suppressiveness, a role of *S. caviscabies* in disease suppression has never been shown. Goyer and Beaulieu (1997) listed S*. caviscabies* among the *Streptomyces* species potentially causing common scab disease in potato. In contrast, Coombs and Franco (2003) reported on a non-pathogenic *S.*

caviscabies strain colonising the endosphere of healthy wheat plants. These authors stressed that the 16S rRNA gene sequences of many *Streptomyces* isolates from the wheat endosphere were similar to the 16S rRNA gene sequence of *S. caviscabies,* but the pathogenicity-related gene, and toxin production (thaxtomin) was not observed in these isolates, indicating that they were non-pathogenic.

Interestingly, the diversity of antagonists isolated from R2A was much higher in soils with history of suppressiveness. By far the highest diversity was determined for antagonists from FR soil. In this soil *Pseudomonas* spp. were less dominant, *Streptomyces* and *Stenotrophomonas* spp. were found to share dominance with *Pseudomonas* spp. In general, the proportion, taxonomic composition, and diversity of antagonistic bacteria were found to be specific for each of the six soils examined in this study. While some genera such as *Pseudomonas*, *Bacillus* and *Streptomyces* were isolated from all or almost all soils, other genera were specific to certain sites. It is important to notice, however, that this sort of analysis by no means represents a robust picture of the community composition of antagonists of each site. Despite our isolation efforts, the final number of antagonists retrieved from R2A (106) is rather limited and does not allow a comprehensive coverage of the actual diversity of antagonists at each site. In spite of this, Figure 3.3 is a highly suited graphic representation of the overall data gathered on antagonists by means of R2A plating and permits the observation of major differences between the soils studied according to the acquired data. The outcome implicit in Figure 3.3 is that the consortium of bacteria able to antagonize soil-borne pathogens in these soils may differ, probably because each site harbors its particular microflora. Indeed, DGGE analyses show that the bacterial community composition in the soils evaluated in this study is different (Lembke et al., unpublished results). Taken these observations together, it is appealing to consider that antagonistic activity towards fungal phytopathogens is a

feature potentially driven by different assemblages of bacteria in each of the soils studied. Moreover, our data indicate that the portion of the bacterial community with antagonistic potential retrieved from suppressive soils, especially in France, is highly diverse. Representatives of both commonly- (e.g. *Pseudomonas*, *Streptomyces*, *Bacillus*,) as well as less frequently reported (e.g. *Dyella*, *Ochrobactrum*, *Brevibacillus*) soil antagonists were observed in our collection of bacterial isolates with antagonistic activity towards *R. solani* and/or *F. oxysporum*. Such diversity of organisms potentially able to play the same role (antagonism) in soil is an indication of functional redundancy in this system.

No correlation between antagonism of a specific fungus and the tested phenotypic traits (enzymes and siderophores productions) for the different soils was found. However, a high abundance of bacterial antagonists that produced proteolytic enzymes and synthesized siderophores was observed in all soils. The role of proteolytic enzymes and siderophores produced by antagonistic bacteria and fungi in biocontrol of plant pathogens have been clearly demonstrated (de Boer et al., 2003; Manwar et al., 2004; Siddiqui et al., 2005; Cao et al., 2005). Dunne et al. (2000) showed that overproduction of extracellular protease in the mutant strains of *Stenotrophomonas maltophilia* W81 resulted in improved biocontrol of *Pythium ultimum*. In our study we found a positive correlation between siderophore production and inhibition of *Ralstonia solanacearum* among antagonistic *Pseudomonas* spp. The same trend was observed by Costa et al. (2006a) when characterizing *Pseudomonas putida* strains isolated from the rhizosphere of maize grown in Brazil. Such indications that siderophore production by *Pseudomonas* spp. might play a role in *R. solanacearum* suppression tie in very well with the report of Jagadeesh et al. (2001), where the involvement of the fluorescent siderophore production by fluorescent *Pseudomonas* spp. in the suppression of bacterial wilt

disease caused by *Ralstonia solanacearum* in tomato plants was demonstrated using fluorescent siderophore-deficient mutants.

CHAPTER 4

CHAPTER 4

Diversity of *Pseudomonas* specific-*gacA* gene among culturable antagonistic *Pseudomonas* isolates and in the bulk soils using PCR-DGGE analysis, and the detection of antibiotic producing genes

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Abstract

In this study, a collection of *Pseudomonas* spp. with *in vitro* antagonistic activity towards *Fusarium oxysporum* and *Rhizoctonia solani* retrieved from six European soils (France, the Netherlands, Sweden, the United Kingdom, and Germany-Braunschweig and -Berlin) with and without prior history of disease suppression was screened by PCR-Southern blot hybridisation for the presence of the genes involved in biosynthesis of 2,4-diacetylphloroglucinol (*phlD*), phenazine (*phzCD*), pyrrolnitrin (*prnD*), and pyoluteorin (*pltC*). *Pseudomonas* spp. containing the *phlD* gene (*phlD*$^+$) were isolated from all sites irrespective of their history of disease suppression. However, the highest number of *phlD*$^+$ *Pseudomonas* isolates (19 of 32) was retrieved from a suppressive soil in Sweden and they also contributed to 50% of the *Pseudomonas* antagonists isolated from this soil. This results suggest that 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. belonged to the dominant pseudomonads populations in Swedish soil and thus might likely play an important role in the natural disease suppression of the Swedish soil. *Pseudomonas* isolates carrying the *prnD* gene were scarcely represented. Antagonists contained neither the *phzCD* nor the *pltC* genes. PCR-DGGE analysis of *gacA* gene fragments obtained from genomic DNA of the antagonistic *Pseudomonas* revealed that the gene was highly diverse among the antagonists originating from each site. Likewise, in the bulk soils, where the antagonists were isolated, the population of *Pseudomonas* carrying the *gacA* gene was diverse and site specific. When *gacA* types derived from the *Pseudomonas* antagonists were linked with the *gacA*-DGGE profiles obtained from TC-DNA of bulk soils only few *gacA* types belonging to the *Pseudomonas* antagonists were represented in their corresponding *gacA* community patterns.

1. Introduction

Bacteria belonging to the genus *Pseudomonas* are one of the best-studied bacterial groups in soil. The genus is notable because it comprises species that are of agricultural important, like plant growth promoters and biocontrol agents and in addition, plant-, animal- and human- pathogenic species (Bossis et al., 2000; Raaijmakers et al., 2002; Weller et al., 2002). *Pseudomonas* spp. produce many different types of metabolites, such as volatiles, toxins, cyclic lipopeptides, enzymes and antibiotics, which accord the genus the ability to compete effectively, and microbial fitness to survive in most environments (Haas & Keel, 2003; Paulsen et al., 2005; Raaijmakers et al., 2006).

In many strains of *Pseudomonas*, expression of genes involved in biosynthesis of these secondary metabolites and extracellular enzymes is positively controlled by a two-component regulatory system, GacA/GacS. The membrane-bound sensor kinase protein (GacS) recognizes yet-unknown environmental stimuli and in tune activates the cytoplasmic response regulator protein (GacA), which triggers the expression of genes controlled by the system (Heeb and Haas 2001). In plant-beneficial *Pseudomonas* spp. the system is necessary for the regulation of genes involved in biocontrol factors, which protect plants from harmful effects of phytopathogens. Such biocontrol factors include production of cell wall degrading enzymes (cellulase, extracellular protease and chitinase) and the biosynthesis of secondary metabolites including pyoluteorin (PLT), pyrrolnitrin (PRN), phenazine (Phz), 2,4-diacetylphloroglucinol (2,4-DAPG) and hydrogen cyanide (Duffy and Défago 2000; Heeb and Haas 2001; Zhang et al., 2001; Fogliano et al., 2002; Haas and Keel, 2003). The detection and characterization of PLT, PRN, Phz, and 2,4-DAPG producers have been greatly enhanced through the use of PCR methods, and the design of specific primers and probes targeting the genes involved in the

biosynthesis of these metabolites (Raaijmakers et al., 1997; de Souza and Raaijmakers, 2003).

The genotypic diversity within microorganisms which possess similar antagonistic traits offers a largely untapped resource for understanding the mechanisms involved in biological control of soil-borne pathogens (de Souza et al., 2003a, Raaijmakers and Weller, 2001). Thus, the knowledge of the *gacA* gene diversity among antagonistic *Pseudomonas* isolates and of the occurrence of a given antagonistic trait among isolates which share similar *gacA* genotypes may further enhance our understanding of the relationship between different *gacA* genotypes and antagonistic function. To date, primers targeting *gacA* gene in *Pseudomonas* spp. have been designed and the gene proposed as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples (de Souza et al., 2003c). However, the diversity of this gene among antagonistic *Pseudomonas* isolates and the effect on their antagonistic traits have been addressed by very few authors. Recently, Costa et al. (2007) developed a novel PCR-DGGE system to assess *Pseudomonas*-specific diversity of *gacA* gene fragments amplified from whole-community bulk and rhizosphere soil DNA. The method has been shown to offer advantages over typical 16S rRNA gene-based analysis and has been proven useful in characterizing *Pseudomonas* isolates antagonistic towards the fungal pathogen *Verticillium dahliae*.

The objectives of the present study were to (i) screen 103 *Pseudomonas* antagonists representing different ecological zones in Europe for the presence of the genes involved in the biosynthesis of the antibiotics 2,4-DAPG, PRN, PLT and Phz, whose regulation by the GacS/GacA system has been clearly demonstrated in pseudomonads with biocontrol activity (Heeb and Haas 2001; Haas and Keel, 2003 Zhang et al., 2001), (ii) determine the diversity of *Pseudomonas*-specific *gacA* gene

among these antagonists by PCR-DGGE analysis and to assess the discriminating power of this system with BOX-PCR fingerprinting, (iii) to determine whether *Pseudomonas* isolates that possess similar *gacA*-DGGE mobilities also share common antagonistic traits (i.e. relationship between the different *gacA* genotypes and antagonistic function) (iv) to determine whether the different isolate-derived *gacA*-DGGE types can be linked with dominant bands in the *gacA* community DGGE profiles of their corresponding soil of isolation.

2. Materials and Methods

2.1. Bacterial strains and soil samples

Approximately 1,788 bacterial isolates from suppressive soils and non-suppressive soils which originated from France (FR), the Netherlands (NL), Sweden (SE), the United Kingdom (UK), and two locations in Germany, Berlin (G-BR) and Braunschweig (G-BS) were screened in our previous studies for antagonistic activity towards *Rhizoctonia solani* AG3 and *Fusarium oxysporum* f. sp. *lini* foln3. In all, 327 antagonists were retrieved and were screened for potential antagonistic traits such as chitinase, glucanase, protease, cellulase activity and siderophores production (Adesina et al., 2007). In the present study, 103 of these 327 (FR=9; NL=32; SE=35; UK=9; G-BR=3; G-BS=15) antagonistic strains, which represented different species of the genus *Pseudomonas* according to fatty acid methyl ester analysis and/or 16S rRNA gene sequencing, were selected for further characterization (Table 4.1). Stock cultures of all strains were stored in Luria Bertani broth plus 20% glycerol at −80℃. Soil samples collected from these sites were also used for further study in the present work. Detailed information on soil sampling and bacterial isolation procedures were described by Adesina et al. (2007).

Table 4.1. Number of different species of *Pseudomonas* antagonists (according to identification by FAME and 16S rRNA sequencing) from each site investigated in this study

Operational taxonomic unit (OTU)	FR	NL	S	UK	G-BR	G-BS	total
P. putida	4 (1)	12	5		1 (1)		22
P. fluorescens	4 (3)	16 (15)	25 (21)	4 (4)	2 (2)	7 (7)	58
P. jessenii				5 (5)		4 (7)	9
P. fulgida			1 (1)				1
P. alcaliphila			1 (1)				1
P. cannabina		1 (1)					1
P. lutea			1 (1)				1
P. marginalis						1 (1)	1
P. chlororaphis						1 (1)	1
P. agarici		1					1
P. veronii	1						1
P. moraviensis		1 (1)					1
P. taetrolens		1 (1)					1
P. syringae-glycine			1				1
Pseudomonas sp.			1			2 (2)	3

Numbers in parentheses are numbers of isolates that could be assigned to a given species based on partial 16S rRNA gene sequencing.

2.2. DNA extraction from bacterial isolates

A single colony of each isolate was streaked on R2A plate and incubated for 48 h at 28^{o} C; cell mass was re-suspended in 0.85% NaCl and centrifuged at 14,000 x *g* for 2 min. To obtain crude cell lysates, the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) was used. DNA extraction was performed using the Ultra Clean TM15 DNA Purification Kit (MoBio Laboratories, Carlsbad, CA, USA). DNA yields were checked on UV light (254 nm) after agarose gel electrophoresis and ethidium bromide staining.

2.3. DNA extraction from bulk soils

The soil samples were processed for DNA extraction with BIO 101 extraction kit (Q.BIOgene, Carlsbad, CA) according to the protocol supplied by the manufacturer.

Following extraction, final DNA purification was performed with the GENECLEAN Spin kit (Q.BIOgene, Carlsbad, CA). DNA yields were checked after electrophoresis on agarose gels stained with ethidium bromide under UV light. DNA was quantified visually by comparison to a 1-kb gene-ruler^{TM} DNA ladder (Fermentas, St. Leon-Rot, Germany) applied on the agarose gels. Extracted community DNA was used for the subsequent PCR reactions after differential dilution of the samples to approximately 1 to 5 ng DNA.

2.4. PCR amplification of the genes involved in antibiotic production

All *Pseudomonas* isolates were screened for the presence of the biosynthetic loci involved in the production of four antibiotics commonly found in *Pseudomonas* strains with biocontrol activity (2,4-DAPG, PRN, Phz, PLT), using specific PCR amplification from genomic DNA. Primers Phl2a/Phl2b, targeting the *phlD* biosynthetic locus, and PCA2a/PCA3b targeting the *phzCD* locus, were described by Raaijmakers et al. (1997) (Table 4.2) and used for the PCR detection of one of the six genes in 2,4-DAPG and two of the nine genes in Phz biosynthetic cluster respectively. PCR amplification of genes in PRN and PLT biosynthetic clusters was performed using the specific primers PRND1/PRND2 and PLTC1/PLTC2 respectively as described by de Souza and Raaijmakers (2003) (Table 4.2). PCR conditions for each reaction were performed as described by the authors. The reference strains *P. fluorescens* CHA0 (for *phlD*, *prnD* and *pltC*) and *P. fluorescens* 2-79 (for *phzCD*) were used as positive controls.

Table 4.2. Primers used for PCR amplification in this study

Gene	Primer	Sequence	Size	Reference
Pyrrolnitrin	prnD1 prnD2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	786 bp	de Souza and Raaijmakers (2003)
Phloroglucinol	PhI2a PhI2b	GAGGACGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	745 bp	Raajmakers et al. (1997)
Phenazine	PCA2a PCA3b	TTGCCAAGCCTCGCTCCAAC CCGCGTTGTTCCTCGTTCAT	1150 bp	Raajmakers et al. (1997)
Pyoluteorin	pltC1 pltC2	AACAGATCGCCCCGGTACAGAACG AGGCCCGGACACTCAAGAAACTCG	438 bp	de Souza and Raaijmakers (2003)
Repetitive genomic sequences	BOXA1R	5′-CTACGGCAAGGCGACGCTGACG- 3′		Rademaker et al. (1999)
GacA	gacA-1F gacA2	TGATTAGGGTGYTAGTDGTCG MGYCARYTCVACRTCRCTGSTGAT	599 bp	Costa et al. (2007) de Souza et al. (2003c)
	gacA-2R gacA-1F-GC-clamp	GGTTTTCGGTGACAG GCA CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGGGATTAGGGTGCTAGTGGTCGA	575 bp	Costa et al. (2007)

2.5. Southern hybridization

To confirm specificity of the PCR products generated from the amplification of the antibiotic biosynthetic *loci*, Southern blot hybridization was performed. The *phlD*, *phzCD* and *pltC* probes were generated from PCR products (745 bp, 1150 bp and 438 bp respectively) amplified from *P. fluorescens* CHA0. The *prnD* probe was derived from PCR products (786 bp) of *P. fluorescens* 2-79. PCR products from the reference strains were excised from the agarose gel and labelled using DIG-labelled dUTP as recommended by the manufacturer (Roche). Southern blotting was done according to Sambrook et al. (1989). PCR products from genomic DNA of all isolates were electrophoresed on agarose gels and subsequently transferred onto HYBOND N nylon membranes (Amersham-Pharmacia Biotech). Hybridisation was performed under conditions of medium stringency following the protocol published by Fulthorpe et al. (1995). Hybridized probes were detected by using a DIG luminescent detection kit (Roche) as specified by the manufacturer.

2.6. Genotypic diversity among isolates by BOX-PCR amplification

BOX-PCR fingerprints obtained from amplification of genomic DNA extract of the antagonists were used for isolate characterization. The amplification was done with BOXA1R primer according to Rademaker et al. (1999) (Table 4.2). BOX-PCR profiles were compared using the software package GelCompar II version 5.6 (Applied Maths, Kortrijk, Belgium). A similarity matrix between the bands of the fingerprints was calculated based on Pearson correlation indices. Dendrograms were created with the unweighted pair-group method using arithmetic averages (UPGMA) linkage. Distinct BOX-PCR groups were defined by similarity coefficient of 70%.

2.7. PCR amplification of *Pseudomonas* specific *gacA* gene fragments for DGGE analysis

Amplification of the *Pseudomonas* specific *gacA* gene fragment (575 bp) from genomic DNA obtained from the *Pseudomonas* isolates and community DNA from the bulk soils was carried out using a nested PCR approach. The primer pair gacA-1F and gacA2 (Table 4.2) was used in the first PCR assay, followed by a second PCR step with the primer set gacA-1FGC and gacA-2R. The PCR conditions used were described recently by Costa et al. (2007).

2.8. Denaturing gradient gel analysis of *gacA* gene fragments

DGGE analysis was performed with the Dcode System apparatus (Bio-Rad Inc., Hercules, CA). The polyacrylamide gel was a double gradient gel of 6 - 9% acrylamide with 27.5 - 58% of denaturant according to Gomes et al. (2004) (where 100% denaturants contains 7M urea and 40% formamide). Aliquots of PCR samples (1–3 µl) were applied to the DGGE gels, and the run was performed in 1X Tris-acetate-EDTA buffer at 58℃ with a constant voltage of 220 V for 6 h. The DGGE gels were silver-stained, according to Heuer et al. (2001). Computer-assisted analysis of DGGE fingerprints was done using GelCompar II version 5.6 (Applied Maths, Kortrijk, Belgium). The DGGE mobility of *gacA* gene fragments amplified from the DNA of *Pseudomonas* antagonists was checked. A marker based on the mixture of *gacA* PCR products generated from all isolates of each site was loaded on DGGE to detect whether these genotypes match dominant bands of the *Pseudomonas*-specific *gacA* DGGE profiles obtained from PCR-amplified community DNA.

3. Results

3.1. Detection of 2,4-diacetylphloroglucinol, pyrrolnitrin, phenazine and pyoluteorin biosynthetic loci

A total of 103 antagonistic *Pseudomonas* isolates, representing different *Pseudomonas* species based on identification by FAME and/or 16S rRNA gene sequencing, were screened by PCR-Southern blot hybridization for the presence of genes (*phlD*, *prnD phzCD*, *pltC*) involved in biosynthesis of four different antibiotics (2,4-diacetylpholorglucinol, pyrrolnitrin, phenazine and pyoluteorin respectively) commonly found in *Pseudomonas* species with biocontrol activity. Thirty-two in 103 antagonists carried the *phlD* gene and they originated from all sites: the highest number was found in SE (19 of 35) followed by NL (8 of 32), G-BR (2 of 3), FR (1 of 9), UK (1 of 9), and G-BS (1 of 15). Only in four isolates (NL=1, SE=1, UK=1, G-BR=1) the *prnD* gene was detected, two of which were *phlD*$^+$*prnD*$^+$. Genes encoding pyoluteorin (*pltC*) and phenazine (*phzCD*) were not found in any of the *Pseudomonas* isolates. All antagonists carrying the *phlD* and/or *prnD* genes were identified by FAME or 16S rRNA sequencing as *P. fluorescens*, except one *prnD*$^+$ antagonist that had 99% identity with the 16S rRNA gene that is related to *P. putida*. Seventeen of the *phlD*+ antagonists identified by 16S rRNA gene sequencing showed between 98-99% close identity to well-known 2,4-DAPG-producing biocontrol strains such as *P. fluorescens* strain Pf-5 (12 antagonists), F113 (4 antagonists) and Pf1 (1 antagonist). One of the two phlD$^+$prnD$^+$ antagonists (K-UK20) had 98% similarity to 16S rRNA gene of *P. fluorescens* strain CHA0 and Pf-5 while the second isolate (K-NL89) displayed 99% identity to both biocontrol strains.

3.2. PCR–DGGE analysis of *Pseudomonas*-specific *gacA* gene fragments

We assessed the presence of *gacA* genes within the assemblage of *Pseudomonas* antagonists by PCR using primers targeting *Pseudomonas* specific *gacA* gene. For few of the antagonists no visible band was observed with ethidium bromide staining from the first PCR products, however, PCR products of expected *gacA* gene fragment size were observed in all investigated *Pseudomonas* antagonists after the second PCR. The melting behaviors observed for PCR-amplified *gacA* gene fragments on DGGE gels were used to determine the diversity of *gacA* types among isolates from each site. *Pseudomonas* antagonists that shared same *gacA* DGGE electrophoretic mobility were also scrutinized for possibility of exhibiting similar antagonistic traits. They were evaluated based on antagonistic traits from our previous study (antifungal activity towards *F. oxysporum* and *R. solani)* and the present study (detection of the antibiotic-encoding genes *phlD* and *prnD*). Other traits, such as activity against *Ralstonia solanacearum*, protease activity and siderophores production were not included, since the vast majority of the *Pseudomonas* antagonists displayed these activities.

Nine *Pseudomonas* antagonists of FR represented six different *gacA* DGGE types (Fig. 4.1a and Fig. 4.2a). Three of the antagonists shared the same *gacA* type (FR-G3). Dual inhibitory activity towards *F. oxsyporum* and *R. solani AG3* (Rhi+Fus+) was a common activity observed among these three isolates, in addition, the only *phlD*$^{+}$ antagonist from FR belonged to this group. Two antagonists represented the *gacA* DGGE type FR-G5 and were both active against *R. solani* AG3. The remaining isolates of FR possessed different *gacA* DGGE mobilities.

The *gacA* fragments amplified from the genomic DNA of *Pseudomonas* antagonists from UK displayed two different DGGE mobilities and were designated as UK-G1 and

UK-G2 (Fig. 4.1b and Fig. 4.2b). For these antagonists *gacA* mobilities displayed by *phlD*$^-$*prnD*$^-$ antagonists was different from *phlD*$^+$*prnD*$^+$ antagonist; eight out of nine antagonists which depicted UK-G1 *gacA* type were *phlD*$^-$*prnD*$^-$ while the only antagonist (K-UK20) represented by UK-G2 *gacA* type was *phlD*+*prnD*+ (a *phlD*$^+$*prnD*$^+$ antagonist which possesses 98% similarity to 16S rRNA gene of *P. fluorescens* strain CHA0 and Pf5). With the exception of one, all UK-G1 *gacA* type antagonists displayed *in vitro* inhibitory activity towards *R. solani* and *F. oxysporum.*

The *gacA* fragments amplified from *Pseudomonas* antagonists from G-BS displayed very diverse electrophoretic mobilities as 15 isolates had 10 different *gacA* types (Fig. 4.1c and Fig. 4.2c). Three antagonists represented by G-BS-G2 were active against *R. solani* whereas another three antagonists described by the *gacA* type G-BS-G6 shared no common traits.

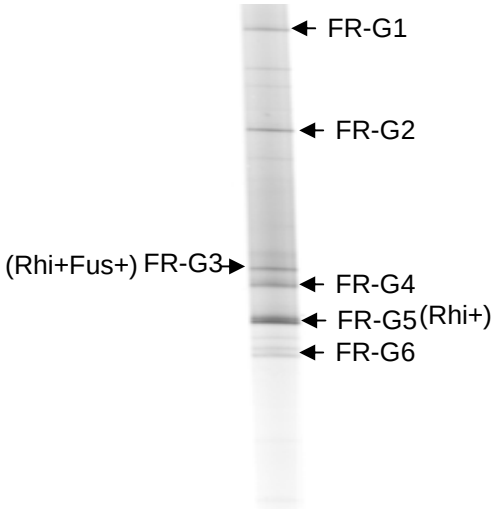
All 35 *Pseudomonas* antagonists from SE represented 14 different *gacA* types (Fig. 4.1d and Fig. 4.2d). Two largest *gacA* DGGE types were found, SE-G5 and SE-G7; each represented by six antagonists. Between one and three isolates could be assigned to the remaining *gacA* DGGE types. Antagonists carrying the *phlD* gene displayed 10 different *gacA* DGGE mobilities and were found in association with *phlD*$^-$*prnD*$^-$ and/or *phlD*$^-$*prnD*$^+$ antagonists. One exception was SE-G7 *gacA* type which comprised only phlD$^+$ antagonists (six isolates) (Fig. 4.1d). These phlD$^+$ antagonists inhibited both *R. solani* and *F. oxysporum,* except two isolates which were active against *R. solani* alone. With the exception of one isolate, all isolates representing DGGE mobility SE-G5 (Fig. 4.1d) showed *in vitro* inhibition towards *R. solani.* Similarly, activity against *R. solani* was found among all isolates represented by the *gacA* types SE-G1 and SE-G2; while isolates with the SE-G9 *gacA* type displayed *in vitro* activity against *F. oxysporum* (Fig. 4.1d).

Thirty-two isolates of NL depicted 11 different *gacA* types, the largest group (11 isolates) belonged to *gacA* type designated NL-G7, followed by another *gacA* type (NL-G9) to which seven isolates could be assigned (Fig. 4.1e and Fig. 4.2e). While the majority of the NL-G7 *gacA Pseudomonas* antagonists inhibited *R. solani AG3*, activity against *F. oxysporum* was mainly displayed by the NL-G9 *gacA* type isolates. Antagonists carrying the *phlD* gene were distributed between these two *gacA* groups, except one *phlD*$^+$ antagonists that possessed different *gacA* type (NL-G4). The three antagonists from G-BR represented two *gacA* DGGE types (Fig. 4.1f and Fig.4.2f). For these antagonists the potential of *gacA* DGGE to differentiate between *phlD*$^+$*prnD*$^-$ and *phlD*$^-$*prnD*$^+$ antagonists was found as two of the antagonists which were *phlD*$^+$ shared the same *gacA* DGGE type (G-BR-G2) while the third isolate, which possessed a different *gacA* type (G-BR-G1), was prnD$^+$.

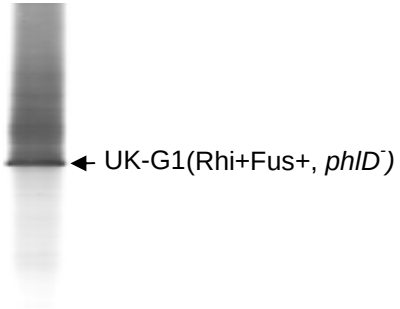
For UK antagonists *gacA* mobilities of phlD$^-$prnD$^-$ antagonists was different from phlD$^+$prnD$^+$ antagonist. Similar differentiation was found between phlD$^+$prnD$^-$ and phlD$^-$prnD$^+$ antagonists from G-BR soil. Whereas for antagonists retrieved from other sites, variable relationship was found between *gacA* mobilities and the presence of antibiotic genes. Likewise, activity towards *R. solani* and /or *F. oxysporum* was variable among *Pseudomonas* antagonist that shared similar *gacA* types. Generally we observed variable relationship between the different *gacA* genotypes and the selected antagonistic functions.

3.3. Assessing discriminating power of the *gacA* DGGE technique using genomic BOX-PCR analysis.

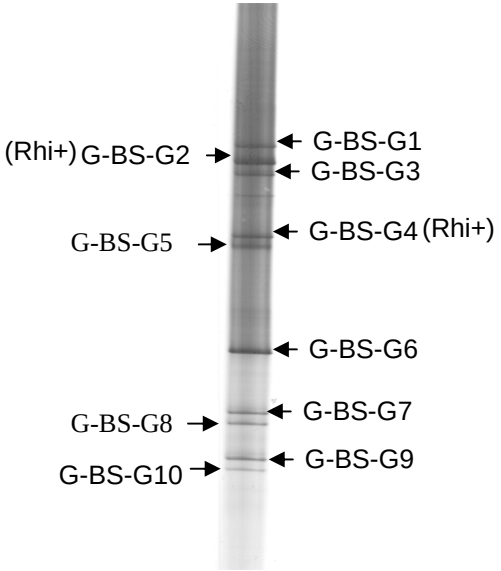
The potential of the *gacA* DGGE technique to make a clear distinction among the different species of *Pseudomonas* antagonists retrieved from each site was checked with discrimination obtained from BOX-PCR fingerprinting methods.


FR-G1
FR-G2
(Rhi+Fus+) FR-G3
FR-G4
FR-G5 (Rhi+)
FR-G6

(a)


UK-G1 (Rhi+Fus+, phlD-)
UK-G2 (phlD+prnD+)

(b)


(Rhi+) G-BS-G2
G-BS-G1
G-BS-G3
G-BS-G5
G-BS-G4 (Rhi+)
G-BS-G6
G-BS-G8
G-BS-G7
G-BS-G10
G-BS-G9

(c)

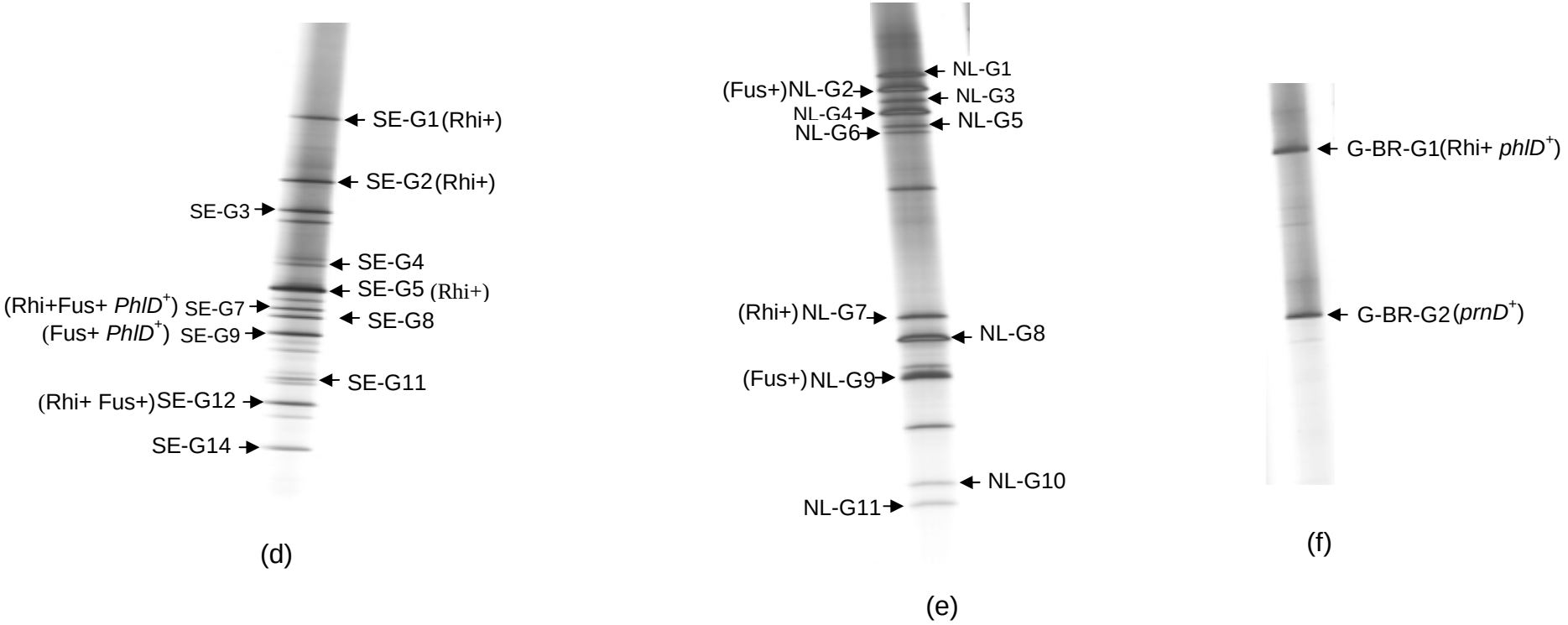
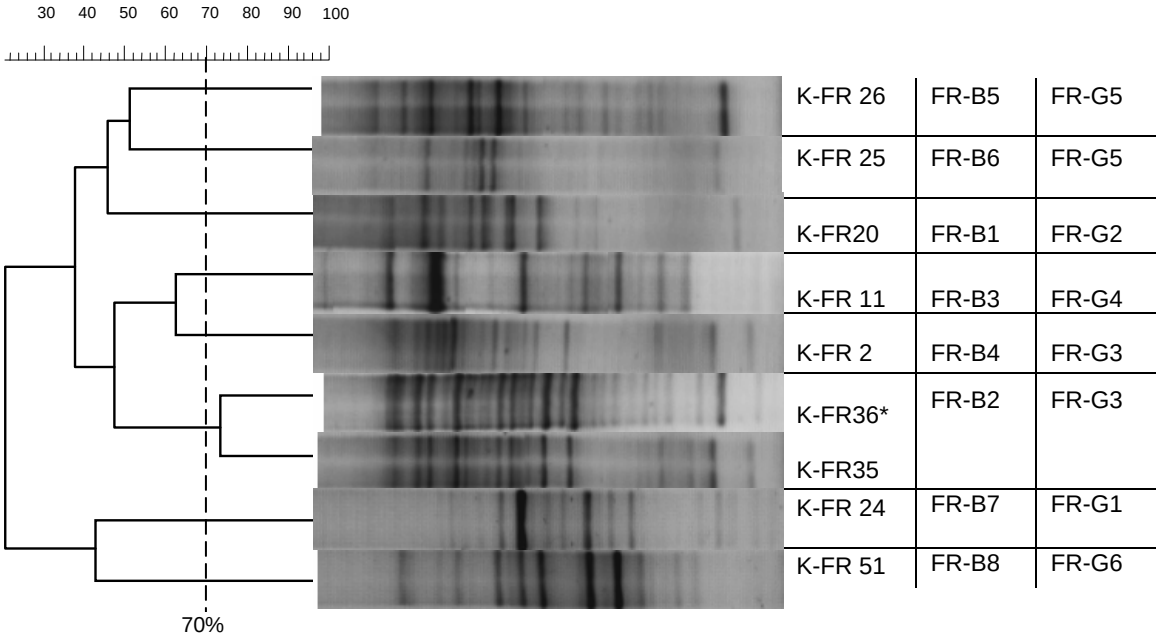
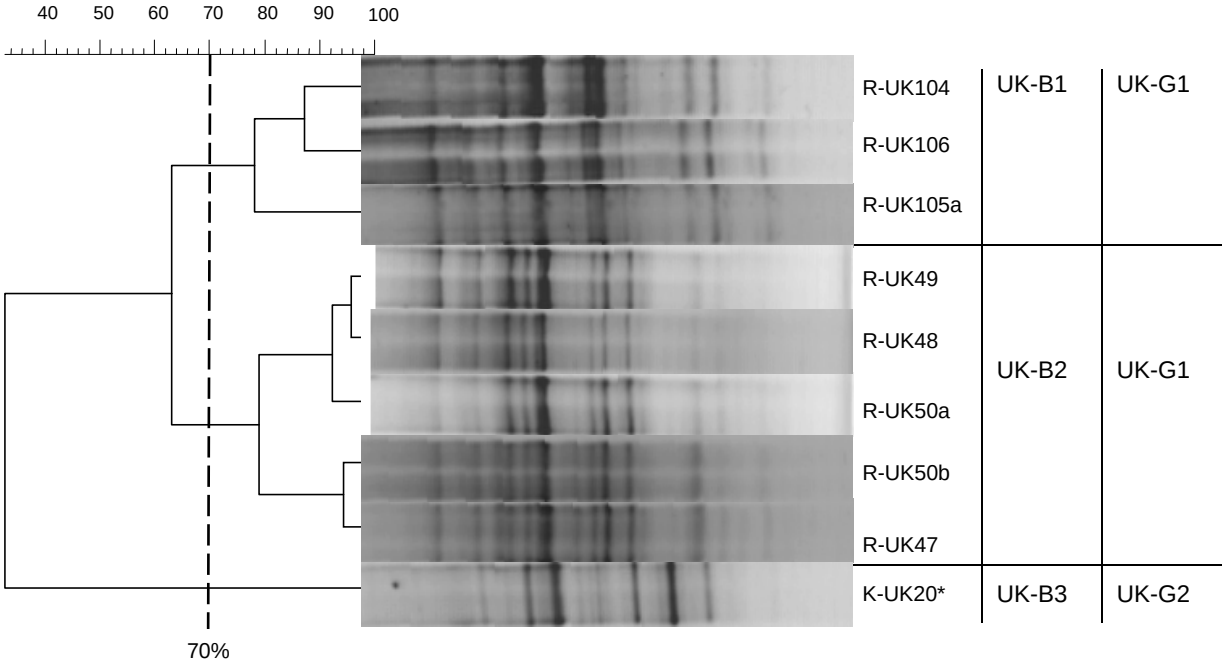


Figure 4.1. DGGE lanes showing different *gacA types* obtained from a mixture of PCR-amplified *gacA* gene fragments of *Pseudomona*s antagonists from (a) FR, (b) UK, (c) G-BS, (d) SE, (e) NL, and (f) G-BR. Beside each band is the *gacA* designation and in brackets are the frequently observed antagonistic traits within a group of isolates sharing the corresponding *gacA* mobility [*In vitro* inhibitory activity towards *R. solani* (Rhi+) and *F. oxysporum* (Fus+), detection of gene encoding 2,4-diacetylphloroglucinol (*phlD*$^+$) and pyrrolnitrin (*prnD*$^+$)].

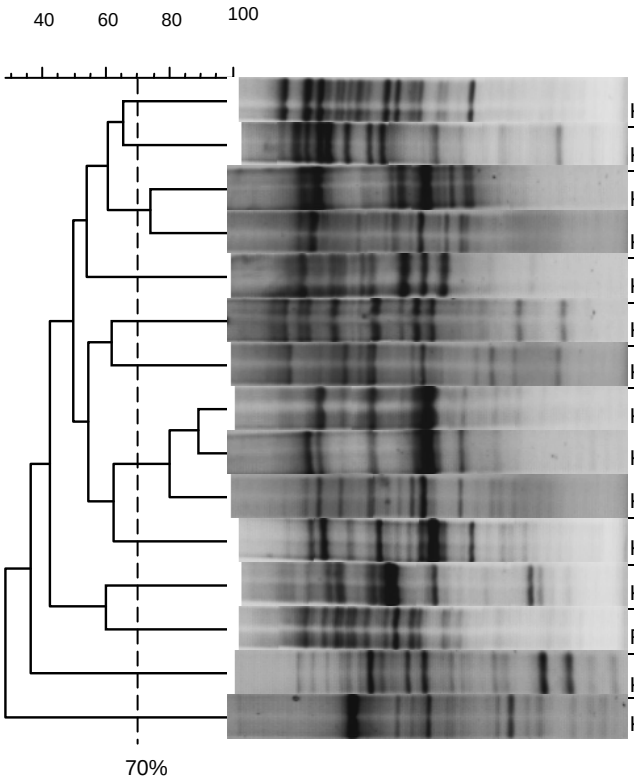
(a)


30 40 50 60 70 80 90 100
70%
K-FR 26 FR-B5 FR-G5
K-FR 25 FR-B6 FR-G5
K-FR20 FR-B1 FR-G2
K-FR 11 FR-B3 FR-G4
K-FR 2 FR-B4 FR-G3
K-FR36* FR-B2 FR-G3
K-FR35
K-FR 24 FR-B7 FR-G1
K-FR 51 FR-B8 FR-G6

(b)

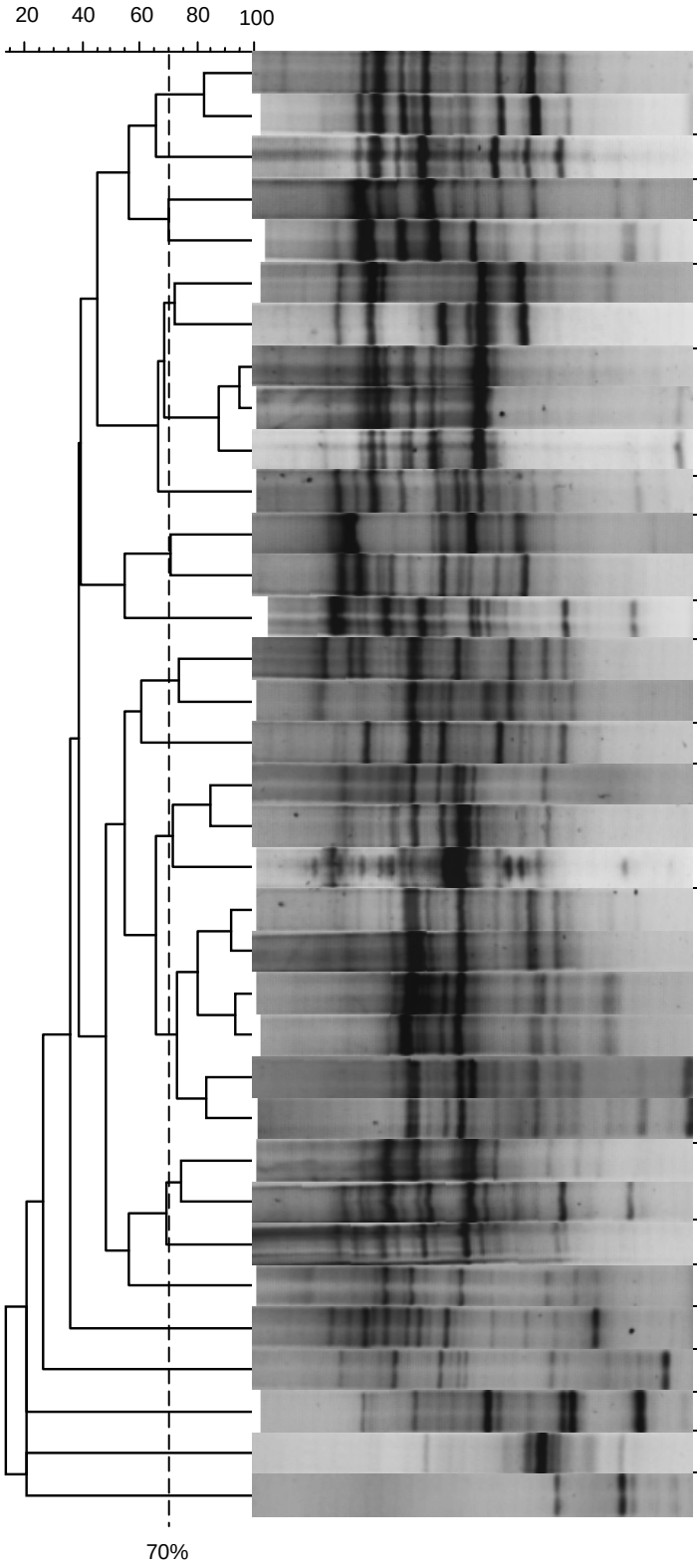

40 50 60 70 80 90 100
70%
R-UK104 UK-B1 UK-G1
R-UK106
R-UK105a
R-UK49 UK-B2 UK-G1
R-UK48
R-UK50a
R-UK50b
R-UK47
K-UK20* UK-B3 UK-G2

(c)



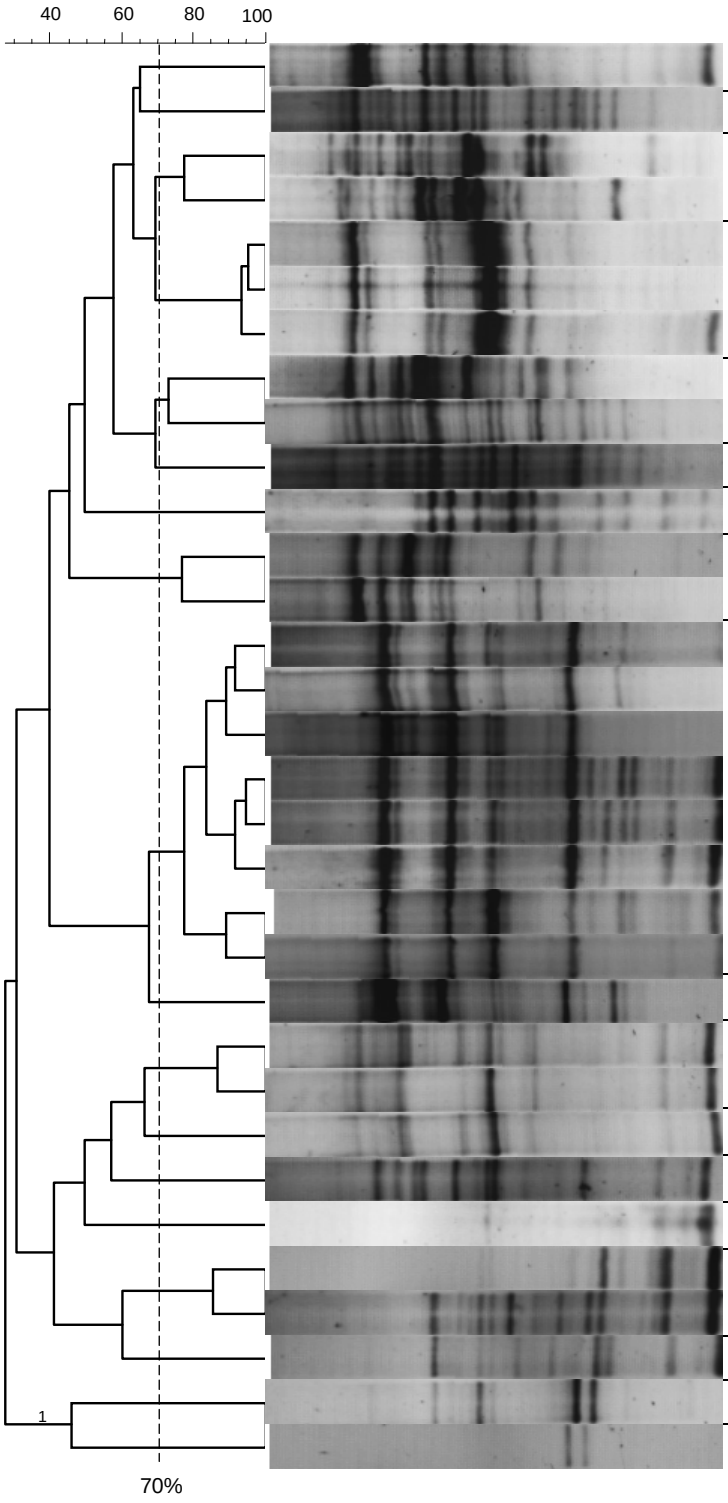
K-G-BS37	GBS-B3	GBS-G8
K-G-BS71	GBS-B4	GBS-G9
K-G-BS47*	GBS-B1	GBS-G6
K-G-BS94		GBS-G4
K-G-BS92	GBS-B5	GBS-G4
K-G-BS3	GBS-B6	GBS-G2
K-G-BS97	GBS-B7	GBS-G6
K-G-BS13	GBS-B2	GBS-G2
K-G-BS6		GBS-G3
K-G-BS99		GBS-G1
K-G-BS5	GBS-B8	GBS-G2
K-G-BS11	GBS-B9	GBS-G5
R-G-BS11	GBS-B10	GBS-G7
K-G-BS2	GBS-B11	GBS-G10
K-G-BS16	GBS-B12	GBS-G6

(d)



K-SE12	SE-B1	SE-G9
K-SE19*		
K-SE18*	SE-B9	SE-G9
K-SE41*	SE-B10	SE-G14
K-SE58*	SE-B11	SE-G5
K-SE11*	SE-B2	SE-G14
K-SE35		SE-G14
K-SE38	SE-B3	SE-G5
K-SE62*		SE-G5
K-SE45		SE-G5
K-SE3*	SE-B12	SE-G8
K-SE16*	SE-B4	SE-G4
K-SE51*		SE-G12
K-SE86	SE-B13	SE-G5
K-SE15*	SE-B5	SE-G3
K-SE74*		SE-G6
K-SE24	SE-B14	SE-G10
K-SE87*	SE-B6	SE-G1
K-SE89		SE-G1
K-SE49		SE-G2
K-SE64*	SE-B7	SE-G7
K-SE76*		SE-G7
K-SE59*		SE-G7
K-SE61*		SE-G7
K-SE65*		SE-G7
K-SE72*		SE-G7
K-SE10*	SE-B8	SE-G12
K-SE90		SE-G6
K-SE73*	SE-B15	SE-G5
K-SE80	SE-B16	SE-G10
K-SE68	SE-B17	SE-G13
K-SE70	SE-B18	SE-G2
K-SE71	SE-B19	SE-G12
K-SE103a	SE-B20	SE-G11
K-SE85	SE-B21	SE-G1

(e)



K-NL28	NL-B8	NL-G5
R-NL63	NL-B9	NL-G7
K-NL55	NL-B1	NL-G4
R-NL37		NL-G7
K-NL41*	NL-B2	NL-G4
R-NL47		NL-G4
K-NL37		NL-G4
K-NL73*	NL-B3	NL-G9
R-NL75		NL-G9
R-NL61b	NL-B10	NL-G1
K-NL29	NL-B11	NL-G2
K-NL74*	NL-B4	NL-G7
K-NL75*		NL-G7
K-NL78*	NL-B5	NL-G7
R-NL70		NL-G7
R-NL94		NL-G7
K-NL88*		NL-G7
K-NL95		NL-G7
K-NL80*		NL-G9
K-NL92		NL-G10
K-NL76		NL-G9
K-NL89*	NL-B12	NL-G7
K-NL91	NL-B6	NL-G9
K-NL99		NL-G9
K-NL100	NL-B13	NL-G9
K-NL72	NL-B14	NL-G11
K-NL5	NL-B15	NL-G8
K-NL84	NL-B7	NL-G7
K-NL96		NL-G6
K-NL39	NL-B16	NL-G3
R-NL85	NL-B17	NL-G2
R-NL86	NL-B18	NL-G2

(f)

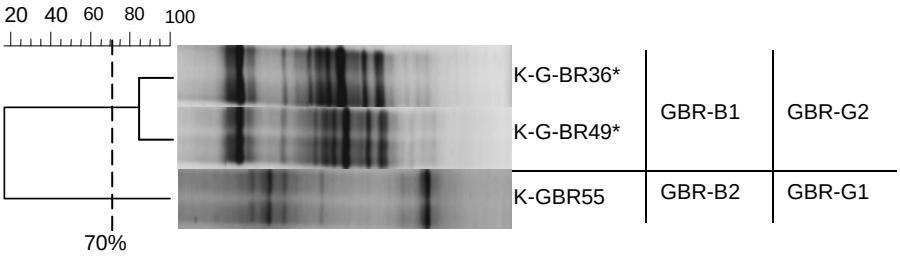


Figure 4.2. Cluster analysis of BOX-PCR fingerprints generated with BOX A1R primer from genomic DNA of *Pseudomonas* antagonists isolated from (a) FR (b) UK (c) G-BS (d) SE (e) NL and (f) G-BR soils. Using GelCompar 5.6, a similarity matrix between the bands of the fingerprints was calculated by Pearson correlation indices and cluster generated by unweighted pair-group method using arithmetic averages (UPGMA). The cut-off of the similarity coefficient used to define distinct genotypic groups was 70%. Isolates designation are listed (first column), distinct grouping of the genomic BOX-PCR fingerprints are labeled (second column), followed by the grouping according to the electrophoretic mobility of the PCR amplified *gacA* fragments from the isolates on DGGE gels. In asterisks (*) are antagonists carrying the *phlD* gene.

Knowing that the resolving power of the BOX-PCR fingerprints is higher than DGGE, especially at the strain level; to make up for this resolution imbalance to a level, the number of *Pseudomonas* species in a given BOX-PCR group was maximized by using a relatively lower similarity index of 70% to assign antagonists into cluster groups. Twenty-one BOX groups were generated from SE (isolates =35), 18 from NL (isolates =32), 12 from G-BS (isolates =15), eight from FR (isolates =9) and three from UK (isolates =9), while DGGE of *gacA* gene fragments detected 14, 11, 10, 6, and 2 unique *gacA* types from each site respectively (Fig. 4.2a, 4.2b, 4.2c, 4.2d, 4.2e and 4.2f).

For some isolates good correlation was found between the two methods. Some antagonists characterized as being distinctly different from others were detected by both methods. These antagonists did not form BOX clusters nor share *gacA* types with any other antagonists. Altogether 19 of such genotypically distinct antagonists were found in all sites: five in each of G-BS and NL, four in FR, three in SE and one

in each of G-BR and UK. They were identified as *P. putida* (8 isolates), *P. fluorescens* (8 isolates), *P. veronii* (one isolate), *P. taetrolens* (one isolate) and *P. alcaliphila* (one isolate). In addition, few isolates which belonged to the same BOX group, possessed the same *gacA* types and which may likely be the same strain were observed. Two of such isolates with 99% close affiliation to *P. fluorescens* were found among G-BR antagonists (G-BR-B1 = G-BR-G2) (Fig. 4.2f). Likewise, seven isolates identified as *P. fluorescens* (SE-B7=SE-G7) were found in SE (Fig. 4.2d). In contrast, a large number of the antagonists, which possessed similar *gacA* types, were separated by BOX-PCR into distinct groups.

3.4. Linking antagonistic potential of *Pseudomonas* to community structure using PCR-DGGE analysis

The diversity of the *Pseudomonas*-specific *gacA* gene in four replicate samples of bulk soils, from which the *Pseudomonas* antagonists were isolated, was evaluated by means of cultivation-independent analysis of whole-community DNA. The *gacA* standard for each site was generated by mixing PCR-amplified *gacA* gene fragments of all *Pseudomonas* antagonists obtained from the same site. In order to assess the dominance of the different *gacA* types represented by the *Pseudomonas* antagonists in their corresponding *gacA* community, the *gacA* standards were loaded next to the *gacA* fingerprints of the bulk soils on the DGGE gels.

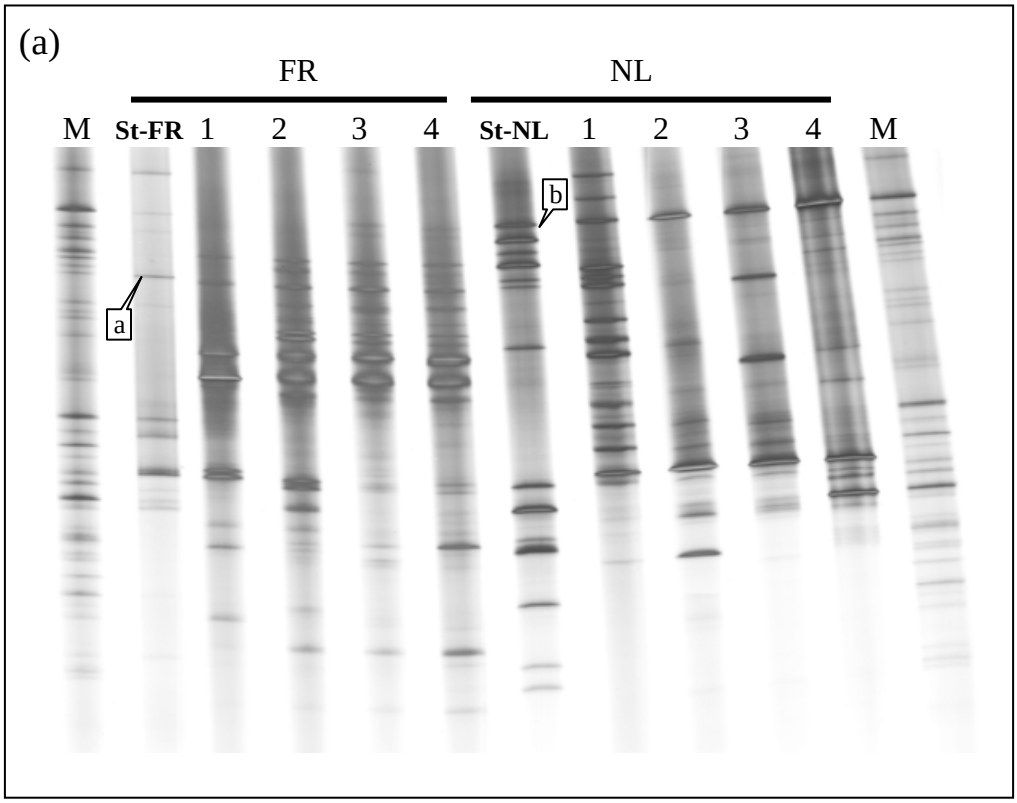
PCR-DGGE analysis of *gacA* gene fragments from the total community revealed a high diversity of *Pseudomonas* carrying the *gacA* gene in all soils. Three out of the four soil replicates from NL (designated 2, 3, and 4; Fig. 4.3a) had the lowest number of *gacA* ribotypes compared to other bulk soils (Fig. 4.3a, 4.3b and 4.3c). Cluster analysis of the *gacA* DGGE fingerprints for the bulk soils showed that the *gacA* community was site specific as replicates from each site were well defined and

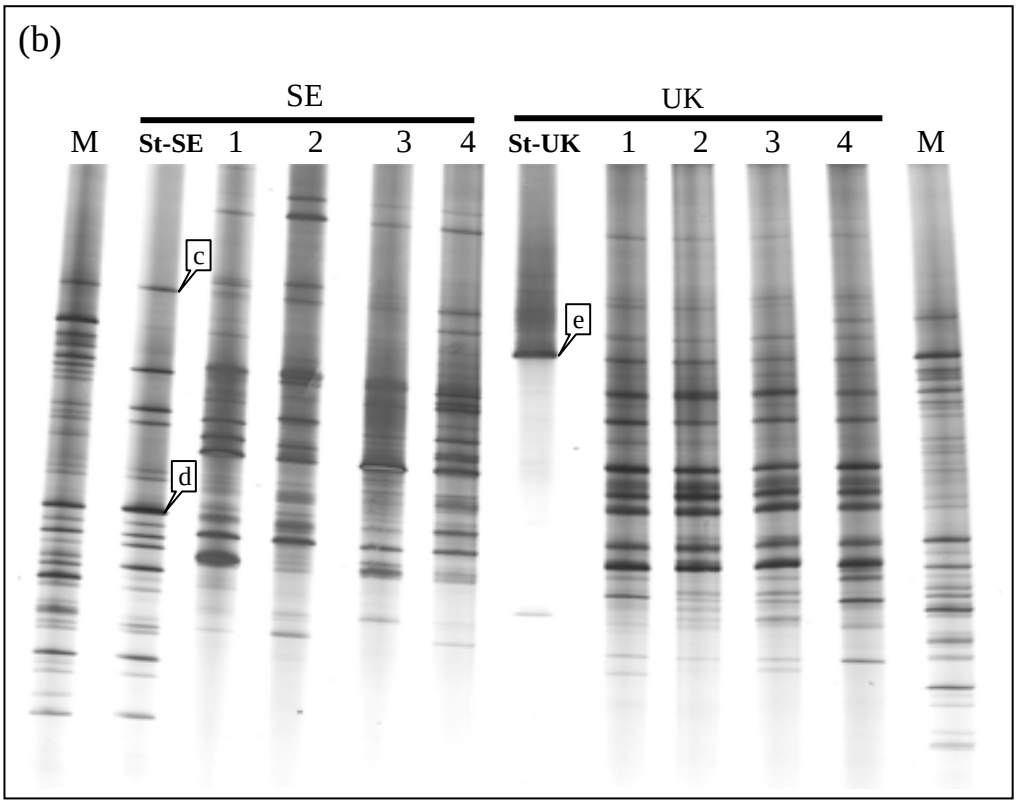
clustered separately from replicates of other sites. An exception was one replicate from G-BR, which formed a cluster with the four replicates from G-BS (Fig. 4.4). When a link between the isolate-derived *gacA* bands and total community-derived *gacA* fingerprints was attempted, some bands belonging to *gacA* fragment of the culturable *Pseudomonas* antagonists shared similar migrating positions with dominant bands in all four replicates of their corresponding *gacA* community. Such isolate-derived *gacA* types were FR-G2, NL-G1, SE-G1, SE-G5, UK-G1, and GBS-G5, whose bands were designated a, b, c, d, e and f respectively (Fig. 4.3a, 4.3b and 4.3c). Of these *gacA* types, UK-G1 and NL-G7 represented the majority of the *Pseudomonas* antagonists obtained from their corresponding site of isolation (Fig. 4.2b and 4.2e). Thus, a good correlation between culture-dependent and -independent analysis of *Pseudomonas* antagonists was revealed for these *gacA* types. The *gacA* DGGE types derived from the *Pseudomonas* antagonists whose bands are less intense or appeared in one or two replicates of the corresponding *gacA* DGGE community were also found (Fig. 4.3a, 4.3b and 4.3c). In contrast, some *gacA* types derived from the antagonists could not be linked to any band in their respective *gacA* DGGE community fingerprints. Generally, we observed that the collection of culturable *Pseudomonas* antagonists derived from each site only corresponded to a minor portion of the "whole" *Pseudomona*s community structure in soil as revealed by *gacA* gene-based analysis.

4. Discussion

Some strains of *Pseudomonas* spp. have received worldwide attention due to their ability to protect plants against a range of agriculturally important fungal diseases. The biological control potential of these strains is partly due to their ability to produce a wide array of anti-fungal metabolites including 2,4-DAPG, Phz, PRN and PLT

(Duffy and Défago 2000; Chin-A-Woeng et al., 2001; de Souza et al., 2003a; Haas and Keel, 2003).





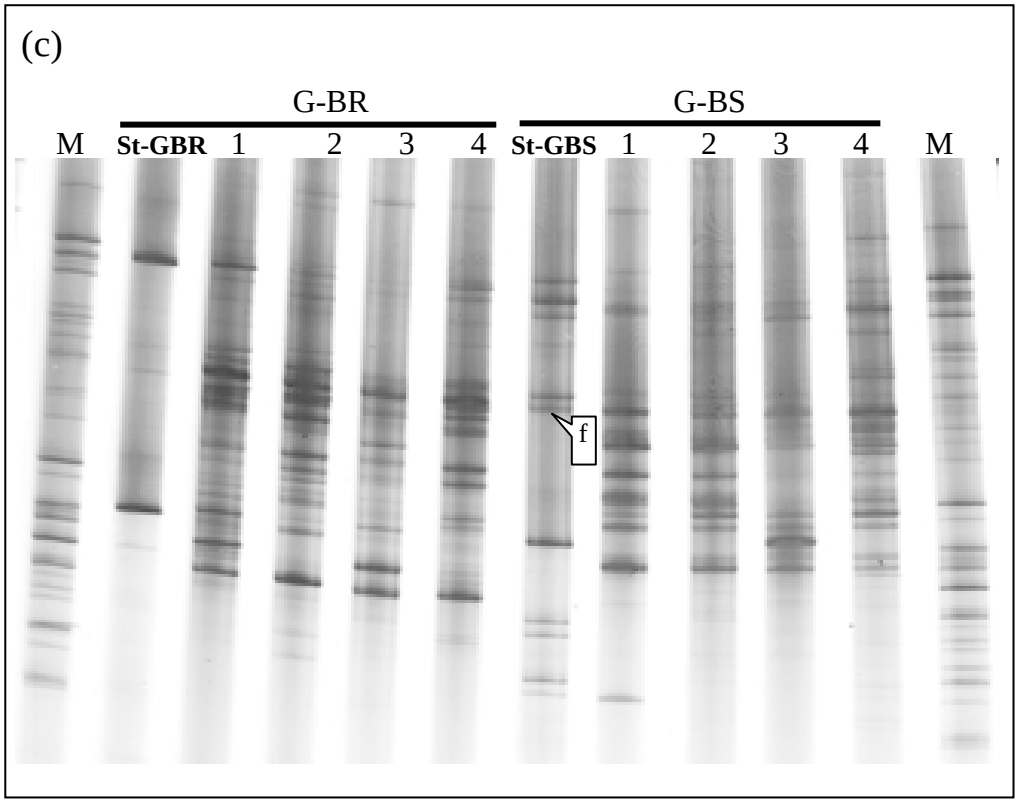


Figure 4.3. Denaturing gradient gel electrophoresis fingerprints of *gacA* gene fragments amplified from bulk soil DNA templates of (a) FR and NL (b) SE and UK (c) G-BR and G-BS. Four bulk-soil replicate samples, designated 1, 2, 3, 4, were analyzed per site. Profile generated by mixing *gacA* gene fragments amplified from the DNA of *Pseudomonas* antagonists isolated from each soil (lane St) was next to their corresponding *gacA* community fingerprints. Lane M represented *gacA* fingerprints of all antagonistic *Pseudomonas* spp. retrieved from all sites.

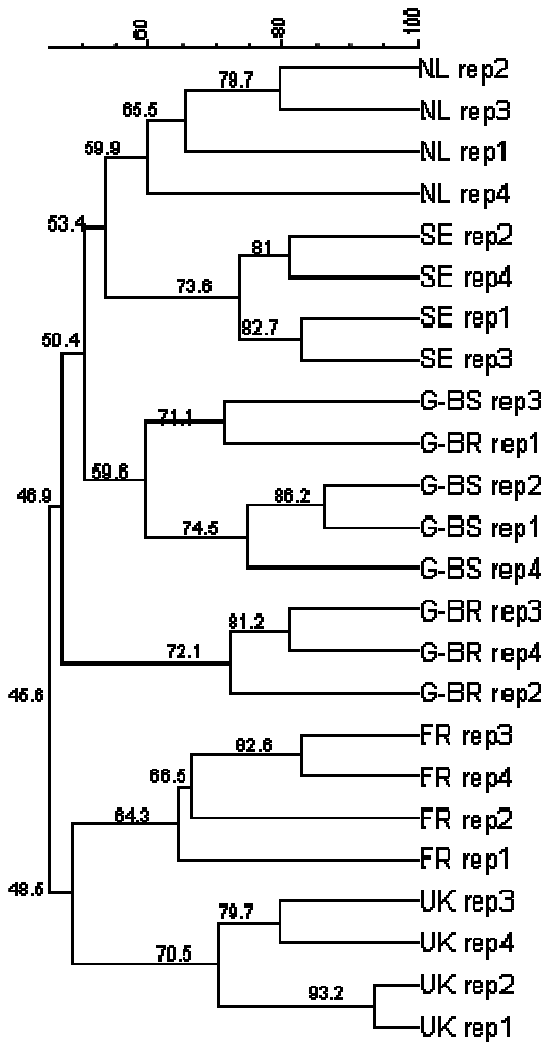


Figure 4.4. Dendrogram showing the relationship of *gacA* DGGE community fingerprints obtained from PCR amplification of bulk soil DNA templates of the six sites (NL, FR, SE, UK, G-BS, and G-BR) using the unweighted pair-group methods with arithmetic average (UPGMA) clustering. Four replicate samples of bulk soil (referred to as rep1, rep2, rep3, and rep4) from each site were analyzed.

Expression of genes involved in biosynthesis of these metabolites is positively controlled by a two-component system, GacS/GacA (Duffy and Défago 2000; Heeb and Haas, 2001). It has been documented that *gacA* gene is conserved among *Pseudomonas* spp. and the reliability of the gene as a complementary genetic marker for detection of this bacterial genus has been proposed (de Souza et al., 2003c). However, very little information is available on the diversity of this gene among antagonistic *Pseudomonas* spp.

This report is a follow-up on our previous study, where a collection of bacterial isolates antagonistic towards *R. solani* and *F. oxysporum* were retrieved from four soils with previous documentation of disease suppression and two soils without documentation of disease suppression (Adesina et al., 2007). Genotypic characterization based on the detection of the genes involved in production of 2,4-DAPG, PRN, PLT and Phz, and PCR-DGGE analysis of the diversity of *gacA* gene among *Pseudomonas* antagonists (n=103) retrieved from this previous study are presented here.

Of the four antibiotic encoding genes investigated in this study, only 2,4-DAPG and PRND encoding genes (*phlD* and *prnD* respectively*)* could be detected among our collection of *Pseudomonas* antagonists. Thirty-two of these antagonists were *phlD*$^+$ and were found in all sites, suggesting the widespread occurrence of *Pseudomonas* antagonists carrying the *phlD* gene. Widespread occurrence of the *phlD* gene among *Pseudomonas* antagonists of worldwide origin was also reported by Raaijmakers et al. (1997). Interestingly, the highest number of *phlD*$^+$ antagonists (n=19) was found in Swedish soil (SE, a soil with known history of suppressiveness to *Plasmodiophora brassicae,* causal agent of club root disease). This number represented 50% of the *Pseudomonas* antagonists active against *F. oxysporum* and *R. solani* retrieved from the Swedish soil (Adesina et al., 2007) and 59% of the *phlD*

positive antagonists obtained from all the six sites. In another study involving cultivation-independent analysis of *phlD* gene in the soils, where these antagonists were isolated, Lembke et al. (in preparation) reported a strong detection of *phlD*-containing *Pseudomonas* population in two of four soil replicate samples obtained from the Swedish soils, whereas their population was below the detection limit in the soil replicates obtained from the other sites, except one replicate from G-BS. These results altogether indicate the high abundance and significant proportion of *phlD*-containing *Pseudomonas* population in SE soil, thus they might likely play an important role in the natural disease suppression that occurs in this soil. The dominance of 2,4-DAPG producers in naturally disease-suppressive soils have been reported by some authors and their contributions to natural disease suppression have been extensively studied (Raaijmakers et al.1997; Weller et al., 2002; de Souza et al., 2003a). Using PCR-DGGE, we observed a high diversity of *gacA* gene among the assemblage of *Pseudomonas* antagonists, as indicated by different migrating positions of the PCR-amplified *gacA* gene fragments obtained from the antagonists of each site. The level of resolution conferred by the strain-specific BOX-PCR technique was used to assess the power of DGGE resolution in differentiating the population of antagonists according to the mobility of the amplified *gacA* gene fragments. Knowing that resolving power of BOX-PCR, which is at strain level, is higher than DGGE, we did not intend to compare the two methods, but to use BOX-PCR as a tool to assess the extent of the discrimination provided with DGGE of *gacA* fragments. However, to compensate for this imbalance in resolution to a level, isolates were assigned into BOX-PCR groups using an arbitrarily chosen but relatively lower BOX fingerprints similarity cut-off of 70%. As expected, the number of genotypes detected by BOX-PCR was higher than *gacA* DGGE for antagonists obtained from each site, except G-BR. Despite this, we still found a good congruence

between the two methods for some of the antagonists. In particular, six antagonists from NL identified as *P. fluorescens* were shown by both methods to possess similar genotype. Similar observation was also found for two *P. fluorescens* from G-BR. In addition, the genotypic distinctness of 19 antagonists identified as *P. putida* (eight isolates), *P. fluorescens* (eight isolates), *P. veronii* (one isolate), *P. taetrolens* (one isolate) and *P. alcaliphila* (one isolate) was revealed by both methods. Nonetheless, a large number of the *Pseudomonas* antagonists that shared similar *gacA* types corresponded to different BOX-PCR groups. For example eight out of nine *Pseudomonas* antagonists isolated from UK shared similar *gacA*-DGGE type, whereas they represented two distinct BOX-PCR groups. The discrepancies observed in the two methods for a larger number of the *Pseudomonas* antagonists arise as a result of the differences in levels of resolution afforded by both methods; basically the number of target gene(s) involved, genome for BOX-PCR versus one gene for *gacA*-DGGE. Moreover, similar *gacA* fragments differing at maximum of four to six nucleotides could possess the same electrophorectic mobility on the DGGE gels, thus, the differences may not be detected by the *gacA*-DGGE technique. Frapolli et al., (submitted) when analysing amplified *phlD* gene from a collection of *P. fluorescens* strains on DGGE, reported that *phlD* sequences differing by as many as seven nucleotides possessed the same migrating position on the DGGE gel. Furthermore, it is well known that multiple bands containing different DNA sequences but similar melting behaviour can co-migrate and assume the same position on the DGGE gel (Muyzer and Smalla, 1998).

We observed variable relationship between the different *gacA* genotypes and the selected antagonistic traits. While for some antagonists that shared similar *gacA* type specific antagonistic traits were found, for other antagonists no common trait was found. For UK antagonists *gacA* mobilities of *phlD*$^{-}$*prnD*$^{-}$ antagonists was different

from phlD$^+$prnD$^+$ antagonist. Similar differentiation was found between phlD$^+$prnD$^-$ and phlD$^-$prnD$^+$ antagonists from G-BR soil. Whereas for antagonists retrieved from other sites, variable relationship was found between *gacA* mobilities and the presence of antibiotic genes. Likewise, activity towards *R. solani* and /or *F. oxysporum* was variable among *Pseudomonas* antagonist that shared similar *gacA* types. 16S rRNA gene sequences of all antagonists carrying the *phlD* gene had close affiliation to *P. fluorescens*, in particular were well known 2,4-DAPG-producing strains like F113, CHA0 and pf-5. However they displayed more than one *gacA* type and BOX-PCR groups, especially in SE, where the frequency of *phlD*$^+$ antagonists was highest. 2,4-DAPG producers, although belonged to a narrow phylogenetic lineage (within the *P. fluorescens* complex), are genotypically diverse. Bergsma-Vlami et al., (2005b) distinguished seven genotypes by DGGE in a collection of 184 *phlD*$^+$ isolates. As many as 18 different BOX-PCR genotypes have been described within a worldwide collection of more than 200 *phlD*$^+$ *P. fluorescens* (McSpadden Gardener et al., 2000; Landa et al., 2002 and Landa et al, 2006). And it is interesting that an extent of this diversity can be reflected by *gacA* characterization as indicated in this study. In contrast, the study of Costal et al. (2007) showed that the *phlD*$^+$ antagonists of *Verticillium dahlae* could be described mainly by one *gacA* type. The reason for this contradiction might be due to a broader representativeness of *phlD*$^+$ antagonists investigated in this study, which originated from different ecological zones in Europe and are genotypically diverse. The *phlD*$^+$ antagonists investigated by Costa et al (2007), on the other hand, were all of German origin and were genotypically conserved according to BOX-PCR fingerprints, probably representing a clonal and competitive rhizosphere *Pseudomonas* population with close affiliation to *P. fluorescens* strain F113.

Cultivation-independent analysis of *gacA* gene in the bulk soils, from where the antagonists were isolated, revealed a high diversity and site-specific *Pseudomonas* *gacA* gene assemblage in each soil. When DGGE fingerprints of *Pseudomonas*-specific 16S rRNA community of each soil (Lembke et al., in preparation) were compared with the *Pseudomonas*-specific *gacA* community, higher number of bands and better resolution were observed with *gacA* community fingerprints. This result corroborates the reports of Adesina et al., (unpublished data), where more bands and better resolution were found with *Pseudomonas*-specific *gacA* community fingerprints of inoculated and non-inoculated lettuce rhizosphere than in their corresponding *Pseudomonas*-specific 16S rRNA communities. Similarly, Costa et al. (2007) reported on the capacity of DGGE analysis of *gacA* gene fragments in differentiating *phlD*$^+$ antagonists from *phlD*$^-$ and *prnD*$^+$ antagonists, a discrimination that was not found with DGGE of *Pseudomonas*-specific 16S rRNA gene fragments. Furthermore, these authors also observed more bands and higher resolving potential in the DGGE profile of bulk and rhizosphere soils with analysis of gacA-based *Pseudomonas* than 16S rRNA-based *Pseudomonas*. These reports all together pinpointed the advantage of DGGE of *Pseudomonas*-specific *gacA* gene over *Pseudomonas*-specific 16S rRNA gene in distinguishing *Pseudomonas* species, particularly in complex environmental samples.

Kisand and Wikner (2003) reported on 3% overlap of bacteria recovered from culture-dependent and PCR-DGGE methods and that some bacteria species detected by the former could not be recovered by the later method. Similar observation was found in our results in an attempt to link culture-derived *gacA* types with the culture-independent *gacA* community in each soil; only few culture-derived *gacA* bands could be matched with bands in their corresponding *gacA* communities. Many of the *gacA* bands obtained from the bulk soil did not possess the same

migrating positions with any culture-derived *gacA* bands. The possible explanations for this may be our strategy of isolation, which used selective medium (Kings B media, from which most of the *Pseudomonas* antagonists were retrieved) and also targeted isolates that are only active against *R. solani* and *F. oxysporum*. It is clear that with this type of isolation strategy, *Pseudomonas* which are not antagonistic towards *R. solani* or *F. oxysporum* have been left out of our collection, but were represented in the *gacA* DGGE fingerprints obtained from the bulk soils. Although it is believed that the genus *Pseudomonas* comprises species that are easy to cultivate (Troxler et al., 1997), some may enter into viable but non culturable state (Mascher et al., 2003), making them to become more resistant to cultivation than the others. This may also be responsible for many of the community-derived *gacA* bands which could not be linked to any of the isolates. Furthermore, some *Pseudomonas* species which are less abundant and would be rather difficult to recover by cultural isolation due to competition with other numerous *Pseudomonas* species were also represented in the *gacA* community. On the other hand, *gacA* bands coming from the isolates but which were not found in the soil profiles could stem from biases associated with PCR amplification, such as low template concentration or genome dosage depending on the DNA extraction method used, which can results to reduction of genotype dominance and composition (Wintzingerode et al., 1997; Kanagawa 2003).

In general, our results indicated a rather higher proportion of *phlD*-containing *Pseudomonas* antagonists in Swedish soil than in the other soils investigated in this study. Thus, this group of beneficial *Pseudomonas* antagonists might likely play a substantial role in the natural disease suppression of the Swedish soil. However, further study will be needed to determine the population of 2,4-DAPG-producing *P. fluorescens* in Swedish soil and their antagonistic effect on the fungal pathogen, *Plasmodiophora brasiccae*, that is naturally suppressed in this soil. We also observed

a high diversity of Pseudomonas-specific *gacA* gene among our collection of antagonists and in the bulk soils. However, the extent of the diversity of this gene within culturable plant-beneficial *Pseudomonas* spp. could not be deduced from the present study, which only investigated representative of few species of this genus. A study involving a broader representativeness of different species of pseudomonad, including pathogenic, plant beneficial and other groups of this genus, will be necessary to determine the extent of the diversity of *gacA* gene within this group of bacteria.

CHAPTER 5

CHAPTER 5

Monitoring rhizosphere competence, biological control and the effects on the soil microbial communities of *in vitro* antagonists towards *R. solani* tested on lettuce plants.

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Summary

Ten *in vitro* antagonists of *Rhizoctonia solani* AG1-IB originating from four different suppressive soils were evaluated for their efficacy in controlling bottom rot disease caused by this fungus on lettuce in growth chamber experiments. The following treatments were done: combined inoculation of each antagonist and *R. solani,* inoculation with *R. solani* and control without inoculation. Although in the first experiment six of the *in vitro* antagonists colonized the rhizosphere of lettuce at comparable rates, only four of the ten isolates (three identified as *Pseudomonas fluorescens* and one as *Pseudomonas jessenii*) significantly decreased disease severity. In subsequent experiments involving the four best antagonists, only *P. jessenii* RU47 effectively and consistently suppressed the pathogen when applied as plant inoculation alone at three weeks after sowing or in combination with seed inoculation. Plate counts on selective media and DGGE of PCR amplified *Pseudomonas*-specific *gacA* gene fragments from total community DNA of inoculated plants confirmed that RU47 established as a dominant *Pseudomonas* population in the rhizosphere of lettuce. The inoculation of RU47 had almost no effects on the *Pseudomonas* communities throughout the seven weeks growing period. Furthermore, PCR-DGGE fingerprints of fungal communities indicated a considerable decrease in the relative abundance of the pathogen in the rhizosphere of lettuce when inoculated with RU47. Strain RU47 produced low molecular weight substance with antifungal activity, protease and siderophores *in vitro,* nonetheless, its exact mechanism of antagonism remains to be explored. Our results suggests that *P. jessenii* RU47 can be considered as a promising biological control agent to suppress *R. solani* AG1-IB in lettuce.

1. Introduction

Rhizoctonia solani Kühn, the anamorph of *Thanatephorus cucumeris* (Frank) Donk, is an ubiquitous widespread soil-borne fungus comprising plant parasitic or saprophytic strains (Ogoshi, 1987). The species affects many important agricultural and horticultural crops worldwide causing diseases such as black scurf, crown rot, and bottom rot. To control the pathogen is difficult because of its wide host range and ability to survive as sclerotia under adverse soil environmental conditions. In practice, control of diseases caused by *R. solani* relies mainly on fungicides, used alone or in combination with other chemicals. However, increasing concern about health and environmental hazards associated with the use of agrochemicals has resulted in the search for viable alternatives to these chemicals. Hence, biological control involving the use of microorganisms that can suppress or antagonize plant pathogens is being considered as a viable substitute or supplement to reduce the use of chemical pesticides (Compant et al., 2005). Over the last decades, bacteria with the ability to suppress plant pathogens have been isolated from different soils; particularly from soils regarded as disease suppressive soils towards *R. solani*, where the inoculum of the pathogen is destroyed or does not survive, or is present but does not induce disease (Mazzola 1999; Weller et al., 2002; Garbeva et al., 2004).

As good as this alternative is, many studies have reported variability in the performance of biological control agents and lack of correlation between *in vitro* inhibition tests and field performance of biocontrol agents (BCA) (Milus & Rothrock, 1997; Schottel et al., 2001). The major contributors to this inconsistency are inherent characteristics of the introduced BCA such as poor root colonization, insufficient production of anti-fungal metabolites at the pathogen infection sites and variable expression of genes involved in disease suppression (Raaijmakers & Weller, 2001). Thus, to validate disease control potential of *in vitro* antagonists, the survival

(rhizosphere competence) and disease suppressive potential need to be assessed in growth chamber or greenhouse experiments that mimic field conditions.

In this study, 10 strains were selected from a collection of bacterial isolates with *in vitro* antagonistic activity towards *R. solani*, which have been previously characterized (Adesina et al., 2007). These strains were isolated from four disease suppressive soils located in France, the Netherlands, Sweden and the United Kingdom. The aims of the work presented here were (i) to investigate the potential of these *in vitro* antagonists to suppress bottom rot disease caused by *R. solani* AG1-IB on lettuce plants *in vivo* (growth chamber experiments, (ii) to evaluate the survival and root colonization efficiency of the antagonists by means of selective plating and to assess antagonists with the best disease suppression for their potential effects on the growth of lettuce plants, (iii) to evaluate the abundance of the most promising antagonist and its effects on the indigenous fungal and bacterial communities in the rhizosphere of lettuce plants using PCR-denaturing gradient gel electrophoresis (DGGE) analysis of 16S and 18S rRNA gene fragments, and the *Pseudomonas* specific global regulatory gene (*gacA*) fragments amplified from total community DNA. This is one of the few studies where the biocontrol efficiency, the survival of biocontrol inoculants and their impact on the indigenous microbial communities were monitored in parallel using a multi-phasic approach.

2. Results

2.1 Pre-screening experiment and *in vitro* characterization of antagonists

From a collection of 248 isolates from suppressive soils, which displayed *in vitro* antagonistic activity towards *R. solani* or *F. oxysporum,* 15 strains were selected based on their *in vitro* activity. To facilitate monitoring of the antagonistic strains after

seed or plant inoculation in greenhouse or growth chamber experiments by selective plating, rifampicin-resistant mutants were generated for all antagonists except the *Streptomyces* strain (AN2-31). For selective isolation of *Streptomyces* sp. AN2-31 a combination of chloramphenicol, nalidixic acid and sulfadiazine added to the medium was used.

To determine the ability of the 15 *in vitro* antagonists to colonize the roots of lettuce plants, pre-screening greenhouse experiment was performed with inoculated lettuce seeds. Cfu per gram of root fresh weight determined four weeks after sowing revealed that at least for nine strains the cfu were above 5 x 10^3 (Table 5.1). One *Streptomyces* isolate, which displayed multi-target activity against different fungi and bacteria *in vitro* but was not included in the pre-experiment, was selected together with the nine strains for the growth chamber experiment. Characterization of these 10 antagonists based on *in vitro* assays from our previous study and the antagonistic activity of the rifampicin resistant mutants towards the representatives of the *R. solani* anastomosis groups AG1-IB (host plant: lettuce), AG2 (host plant: sugar beet) and AG3 (host plant: potato) are summarized in Table 5.1. Seven antagonists (KS16, KS90, KS70, KS74, KF36, KF32b and AN2-31) showed strong *in vitro* inhibitory activity against *R. solani* AG1-IB (the model pathogen used in this study for the growth chamber experiments) with inhibition zones larger than 6 mm while RN86 expressed moderate inhibitory activity of less than 6 mm zone of inhibition. AFNS31 and RU47 showed weak activity against *R. solani* AG1-IB, inhibition zones were less than 3 mm.

Table 5.1. *In vitro* characterization and cfu counts (g^{-1} of fresh root weight) from the pre-screening greenhouse experiment for the ten antagonists selected for growth chamber experiments according to the data obtained from this study and our previous study.

	Antifungal activity				hydrolytic enzymes						Pre-screening greenhouse experiment	
	R. solani *AG1-IB**	R. solani AG2*	*R. solani* AG3	*F. oxyspor*.	Protease	Glucanase	Cellulase	Chitinase	Siderophore.	2,4-DAPG	(Log $_{10}$ CFU g^{-1} of fresh root weight)	Identification by 16S rRNA gene sequencing (% Identity)
KS16	+++	+	+++	++	+	-	-	-	+++	+	4.94 ± 0.36	*P. fluorescens* (100%)
KS90	+++	++	+++	-	+	-	-	-	+++	+	4.29 ± 0.30	*P. fluorescens* (100%)
KS70	+++	++	++	+	+	-	-	-	+++	-	3.65 ± 1.03	P. fulgida (98%)
KS74	+++	++	+++	+	+	-	-	-	+++	+	4.14 ± 0.05	*P. fluorescens* (100%)
KF36	+++	++	+++	-	+	-	-	-	+++	+	4.63 ± 0.42	*P. fluorescens* (99%)
KF32b	+++	-	+++	-	-	-	-	-	+	-	3.42 ± 0.92	*Alcaligenes faecalis* (99%)
AFNS31	+	-	+	+	+	-	-	+	+	ND	4.17 ± 0.75	*Serratia marcescens* (100%)
RN86	++	-	+	+	+	-	-	-	+	-	3.43 ± 0.71	*P. cannabina* (99%)
AN2-31	+++	+	+	+	-	-	-	-	+	ND	ND	*Streptomyces purpureus* (98%)
RU47	+	+	+	+	+	-	-	-	++	-	4.07 ±0.25	*P. jessenii* (99%)

Legend: Zones of inhibition rating of the antifungal activity, + mycelia die back and inhibition zone of 1.0 - 2.9 mm, ++ inhibition zone of 3.0 – 5.9 mm, +++ inhibition zone of 6.0 mm and more, - no activity; *****- *in vitro* assay done in this study; DAPG – detection of gene (*phlD)* involve in biosynthesis of 2,4-diacetylphloroglucinol by PCR-Southern blot hybridization; ND- not determined.

2.2 Growth chamber experiments

The ability of the ten antagonistic bacteria to suppress bottom rot disease caused by *R. solani* AG1-IB on lettuce was evaluated in growth chamber experiments under controlled environmental conditions. The experiment consisted of four consecutive growth chamber experiments. In experiment 1, all ten *in vitro* antagonists against *R. solani* AG1-IB were included. Four antagonists (RU47, KF36, KS16 and KS74) which showed the best disease suppression in experiment 1 were tested in experiments 2 and 3, while only two of the antagonists (RU47 and KF36) with the most promising results were repeated in experiment 4. Survival of inoculants in the rhizosphere of lettuce plants was monitored by dilution plating on selective media in experiments 1, 2, and 3. The growth promoting effects of the inoculants on shoot and root dry mass of lettuce plants in treatments inoculated with each of the antagonists alone (without *R. solani* inoculation) was only investigated in experiments 3 and 4.

2.3 Survival of the antagonists in the rhizosphere of lettuce plants

Despite of similar cell density of bacterial suspension used for seed inoculation (about log 9.0 cfu ml^{-1}), cell densities of antagonists on the seed surfaces were in the range of log 4.81 – 7.81 per seed (data not shown). However, some of the bacteria inoculated on the seeds were actively colonizing the lettuce roots as evidenced by the cfu counts of the inoculant strains determined three weeks after sowing, which ranged between log 5.22 and 7.57 g^{-1} fresh root, for the three of the experiments where cfu was determined (3 WAS; Table 5.2). The cfu per gram of roots for all antagonists decreased with time with a decrease of about one order of magnitude or more at seven weeks after sowing; except for KS16, which increased slightly in experiment 1 (Table 5.2).

Table 5.2. Colonization of lettuce plants (log $_{10}$ cfu g^{-1} of fresh root) by bacterial antagonists at 3, 5 and 7 WAS for experiments 1, 2 and 3.

	3 WAS	5 WAS	7 WAS
Experiment 1			
RU47+Rs	7.17 ± 0.04 cb	6.72 ± 0.15 a	5.92 ± 0.12 cb
KF36+Rs	7.57 ± 0.27 c	6.55 ± 0.22 a	6.63 ± 0.12 c
KS16+Rs	6.39 ± 0.37 ab	6.40 ± 0.55 a	6.58 ± 0.19 c
KS74+Rs	6.57 ± 0.26 ab	6.46 ± 0.13 a	5.82 ± 0.23 cb
KS90+Rs	6.52 ± 0.08 ab	6.81 ± 0.66 a	5.95 ± 0.39 cb
KS70+Rs	6.20 ± 0.53 a	6.21 ± 0.58 a	4.75 ± 0.39 a
AFNS31+Rs	6.85 ± 0.38 ab	6.15 ± 0.33 a	5.53 ± 0.42 ab
KF-32b+Rs	ND	ND	ND
RN86+Rs	6.68 ± 0.02	ND	ND
AN2-31	5.22 ± 3.01	ND	ND
Experiment 2			
RU47+Rs	6.19 ± 0.38 a	5.00 ± 0.33 a	4.88 ± 0.49 a
KF36+Rs	6.55 ± 0.30 a	5.93 ± 0.21 b	5.54 ± 0.44 a
KS16+Rs	6.53 ± 0.07 a	5.48 ± 0.35 ab	5.45 ± 0.24 a
KS74+Rs	5.86 ± 0.47 a	4.96 ± 0.38 a	4.92 ± 0.33 a
Experiment 3			
RU47+Rs	5.82 ± 0.06 a	5.24 ± 0.27	5.62 ± 0.22
KF36+Rs	5.92 ± 0.06 a	ND	ND
KS16+Rs	5.22 ± 0.22 a	ND	ND
KS74+Rs	6.09 ± 0.28 a	ND	ND

CFU of the same experiment followed by the same letter are not significantly different according to Turkey test (P = 0.05). ND – parameter not determined due to the death of nearly all plants, thus no surviving plants for sampling.

At the end of experiment 1, the ability of five of the isolates (RU47, KF36, KS16, KS74 and KS90) to colonize lettuce plants was statistically similar (Table 5.2). Similar results were obtained in experiment 2, which was performed only with four of these isolates (RU47, KF36, KS16 and KS74). Root colonization of isolates KF32b, AN2-31 and RN86 in experiment 1 as well as isolates KF36, KS16 and KS74 in experiment 3, could not be followed until the end of the experiment due to the severity of the disease incidence which resulted in lack of sufficient numbers of surviving plants for sampling after pathogen inoculation.

Despite of a second inoculation of the antagonists done by watering the plants around the stems three weeks after sowing, a slight decrease in the cfu g^{-1} fresh root was observed two weeks after the second inoculation (5 weeks after sowing and two weeks after transplanting). The exception was a slight increase observed at 5 WAS in cfu g^{-1} fresh root of KS16, KS90 and KS70 in experiment 1 (Table 5.2). However, this effect was not observed in other experiments.

To determine the effect of *R. solani* on the survival of RU47, cfu counts of RU47 in the rhizosphere of plants inoculated with and without *R. solani* were compared in experiment 3, cfu counts of RU47 did significantly increase in treatment inoculated with RU47 and *R. solani* (data not shown).

The ability of isolate RU47 to colonize the vascular tissue of lettuce leaves and roots was determined in experiment 3 at 7 weeks after sowing by dilution plating of the suspension of surface-sterilized macerated roots and leaves from lettuce plants inoculated with RU47. We observed no colony growth on the plates, suggesting that the strain did not grow endophytically.

2.4 Suppression of bottom rot disease of *R. solani AG1-IB* by introduced bacterial antagonists

At the end of each experiment a total of 24 plants per treatment was used to evaluate the ability of the inoculated antagonists to suppress bottom rot disease caused by *R. solani* AGI-IB on lettuce plants. Evaluation of disease symptoms was based on the number of surviving plants. Disease severity was assessed with respect to the loss in shoot dry mass compared to the non-inoculated healthy control (Ctrl).

In experiment 1, disease severity was high as evidenced by the low numbers of surviving plants (one of 24 plants) and high reduction in the shoot biomass (indicator of disease severity) in the treatment inoculated only with *R. solani* (Ctrl+Rs) (Figure 5.1 and 5.2). However, also in the treatments inoculated with the strong *in vitro* antagonists KF70, KF32, AN2-31 and medium and weak antagonists RN86 and AFNS31, respectively (Table 5.1) none or low numbers of surviving plants (less than seven of 24) and strongly reduced dry shoot biomass (more than 57%) were found (Table 5.3). This indicates the high pathogenicity of the *R. solani* AG1-IB strain used in this study on lettuce plants. Based on the higher numbers of surviving plants and significantly reduced disease severity (varied between 6 – 25%) obtained with isolates RU47, KF36, KS74 and KS16 in experiment 1 (Figure 5.1 and Table 5.3), experiments 2 and 3 were repeated with these strains. Despite the high level of disease severity, the highest number of surviving plants and lowest disease severity was found in treatments inoculated with RU47 in all experiments (Figure 5.1, 5.2 and Table 5.3), indicating a consistent and strong antagonistic activity of this bacterium towards *R. solani* AG1-IB. Although, KF36 had similar numbers of surviving plants and disease suppression as RU47 in experiments 1 and 2, the disease suppressive effect was not consistent throughout the four experiments. In fact, none or one surviving plant was recovered from treatments inoculated with KF36 in experiments 3

and 4 (Table 5.3). Compared with RU47 and KF36, strains KS16 and KS74 had a much less pronounced control effect on *R. solani* AG1-IB in experiment 2.

To investigate the effect of the time of inoculation on bottom rot disease suppression, isolates RU47 and KF36 were applied as seed treatment or plant treatment alone or as a combination of the two treatments in experiment 4 (Table 5.3). Application of RU47 and KF36 as seed inoculation alone did not suppress bottom rot disease, whereas for RU47 better disease suppression was achieved when applied as plant inoculation alone or as combined seed and plant inoculation (Table 5.3).

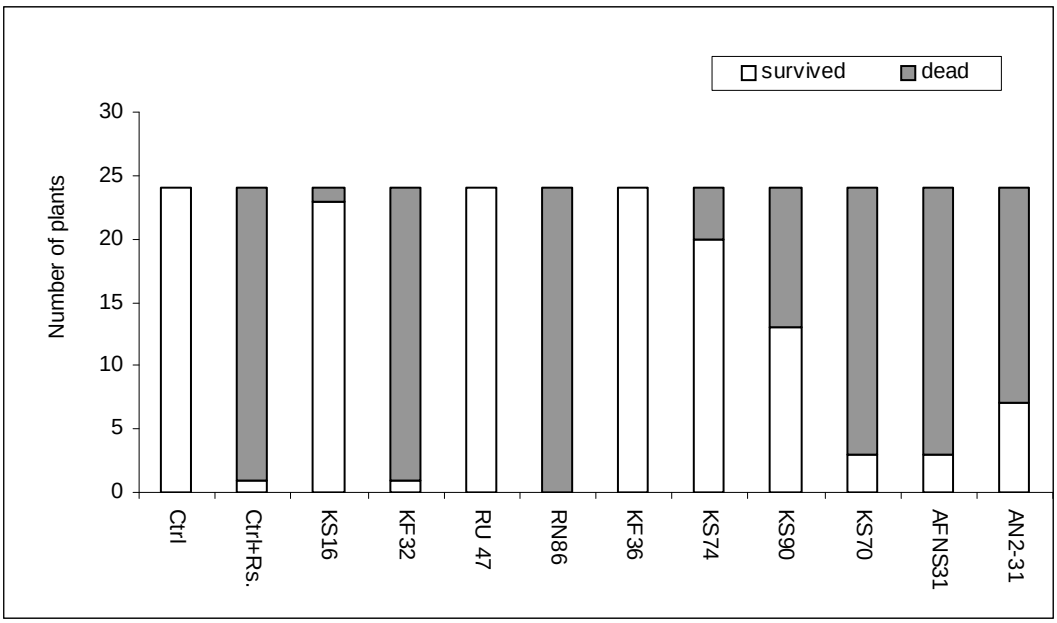

Figure 5.1. Number of surviving versus dead plants after four weeks of *R. solani* AG1-IB inoculation (7 WAS) in experiment 1. The ability of the ten selected antagonists to suppress bottom rot disease of *R. solani* AG1-IB was evaluated based on the number of surviving and dead plants in treatments inoculated with each of the antagonists combined with *R. solani* AG1-IB.

Figure 5.2. Effect of *R. solani* and/or *Pseudomonas jessenii* (the most promising bacterial antagonists*)* on lettuce plants compared with the control plants: (a) Untreated plants (ctrl); (b) sole *R. solani* AG1-IB inoculated plants (Rs); and (c) plants inoculated *with P. jessenii* and *R. solani* AG1-IB (RU47+Rs).

Table 5.3. Number of surviving plants (n = 24) and reduction in shoot biomass in seed and/or plant inoculated treatments

	Experiment 1		*Experiment 2*		*Experiment 3*		*Experiment 4*	
	Surviving plants	Disease severity (%)	Surviving plants	Disease severity (%)	Surviving plants	Disease severity (%)	Surviving plants	Disease severity (%)
Ctrl + Rs	1	82.7	11	43.88	0	78	0	88.39
Seed and plant inoculation								
RU47+Rs	24	10.2*	24	10.0*	17	35.0*	21	19.4*
KF36+Rs	24	6.2*	24	14.0*	1	80.0	0	89.7
KS16+Rs	23	22.0*	19	27.8	2	79.0		
KS74+Rs	20	25.1*	21	23.2	1	77.0		
KS90+Rs	13	58.0						
KS70+Rs	3	74.0						
AFNS31+Rs	3	70.1						
KF32+Rs	1	84.0						
RN86+Rs	0	87.7						
AN2-31+Rs	7	65.7						
Seed inoculation alone								
RU47+Rs							0	89.7
KF36+Rs							0	88.4
Plant inoculation alone								
RU47+Rs							23	8.4*
KF36+Rs							1	89.0

*- Significant differences in comparison with the *R. solani* AG1-IB control treatment (Ctrl+Rs) according to Tukey's test (P = 0.05) for the corresponding experiment

Table 5.4. Growth promoting effects of the best four antagonists on shoot and root biomass for experiment 3 and 4

	Experiment 3		Experiment 4	
Treatment	Shoot	Root	Shoot	Root
Ctrl	2.87	0.74	1.55	0.41
RU47	2.87	0.76	1.24*	0.36*
KF36	3.01	0.74	1.92*	0.48*
KS16	2.64*	0.69		
KS74	2,91	0,78		

*- Significant differences in comparison with the healthy control (Ctrl, - treatment without *R. solani* inoculation) according to Tukey's test (P = 0.05) for the corresponding experiment.

2.5 Growth promoting effects of bacterial antagonist on lettuce plants

The growth promoting effects of the four isolates (RU47, KF36, KS16 and KS74) which showed significant disease suppression in experiment 1 was evaluated in experiment 3, while only isolates RU47 and KF36 were included for similar investigation in experiment 4. The growth promoting effect was determined in treatments inoculated with each bacterial antagonist without pathogen inoculation. The shoot and root dry mass obtained in the inoculated treatments were compared with the non-inoculated healthy control (Ctrl). Inoculation with KF36 promoted shoot biomass in the two experiments, while root growth promotion was only observed in experiment 4 (Table 5.4). Shoot and root biomass in the treatments inoculated with KS16, KS74 and RU47 alone were similar to the healthy control plants in experiment 3 and thus no growth promoting effect was observed for these strains (Table 5.4).

2.6 Treatment effects on indigenous microbial communities

The effect of the pathogen *R. solani* and of the inoculant strain *P. jessenii* RU47 on the relative abundance of the dominant bacterial, fungal and *Pseudomonas* populations in the rhizosphere of lettuce plants was investigated using DGGE analysis of 16S/18S rRNA or *gacA* gene fragments amplified from total community DNA. DGGE profiles were compared between three replicate rhizosphere samples from the non-inoculated healthy control plants (Ctrl), *R. solani* sole inoculated plants (Ctrl+Rs), and plants with combined inoculation of RU47 and *R. solani* (RU47+Rs) at three sampling times (3, 5 and 7 WAS). The results presented are for experiment 3.

The DGGE bacterial community profiles showed complex band patterns with many bands. Detection of strain RU47 in the bacterial rhizosphere patterns of inoculated plants was impossible due to the presence of a band with identical

electrophoretic mobility as 16S rRNA gene fragment amplified from RU47 in the replicates of Ctrl and Ctrl+Rs treatments (Figure 5.3a and 5.3b). This band was much less intense at 7 WAS in the replicates of RU47+Rs (Figure 5.3b). Cluster analysis of the DGGE bacterial community profiles showed that each treatment formed separate clusters at each sampling time (Figure 5.3a and 5.3b). In general, the DGGE patterns of the replicates of each treatment displayed a high similarity. At 3 WAS a clear effect of the seed inoculation could be detected. While the replicates of each treatment were more than 80% similar, the cluster of the seed inoculated treatment shared only about 58% similarity with the control cluster (data not shown). The bacterial patterns at 5 WAS (two weeks after transplanting) displayed a much higher degree of variability and the clustering of the treatments was less pronounced (Figure 5.3a). Seven WAS again pronounced clusters were observed for each treatment. All replicates treated with *R. solani* showed more than 60% similarity with two sub-clusters formed at about 74% for the treatment RU47+Rs and Ctrl+Rs and were clearly separated from the untreated control (Figure 5.3b).

Analysis of the *Pseudomonas* community pattern in the rhizosphere of lettuce plants showed a band with the same mobility as RU47 only in treatments inoculated with the antagonist at 3 and 5 WAS (data not shown), whereas at 7 WAS, the detection of RU47 was not possible because a band which possessed the same electrophoretic mobility as the 16S rRNA gene fragment amplified from RU47 was dominant in all treatments (Figure 5.4). In contrast to the bacterial community profiles, *Pseudomonas* rhizosphere patterns of lettuce plants were less complex with fewer numbers of bands. Aside from a band (Figure 5.4, band a) that was pronounced in the untreated control and RU47 inoculated samples at 7 WAS, the banding patterns did not markedly differ at each sampling time for all treatments.

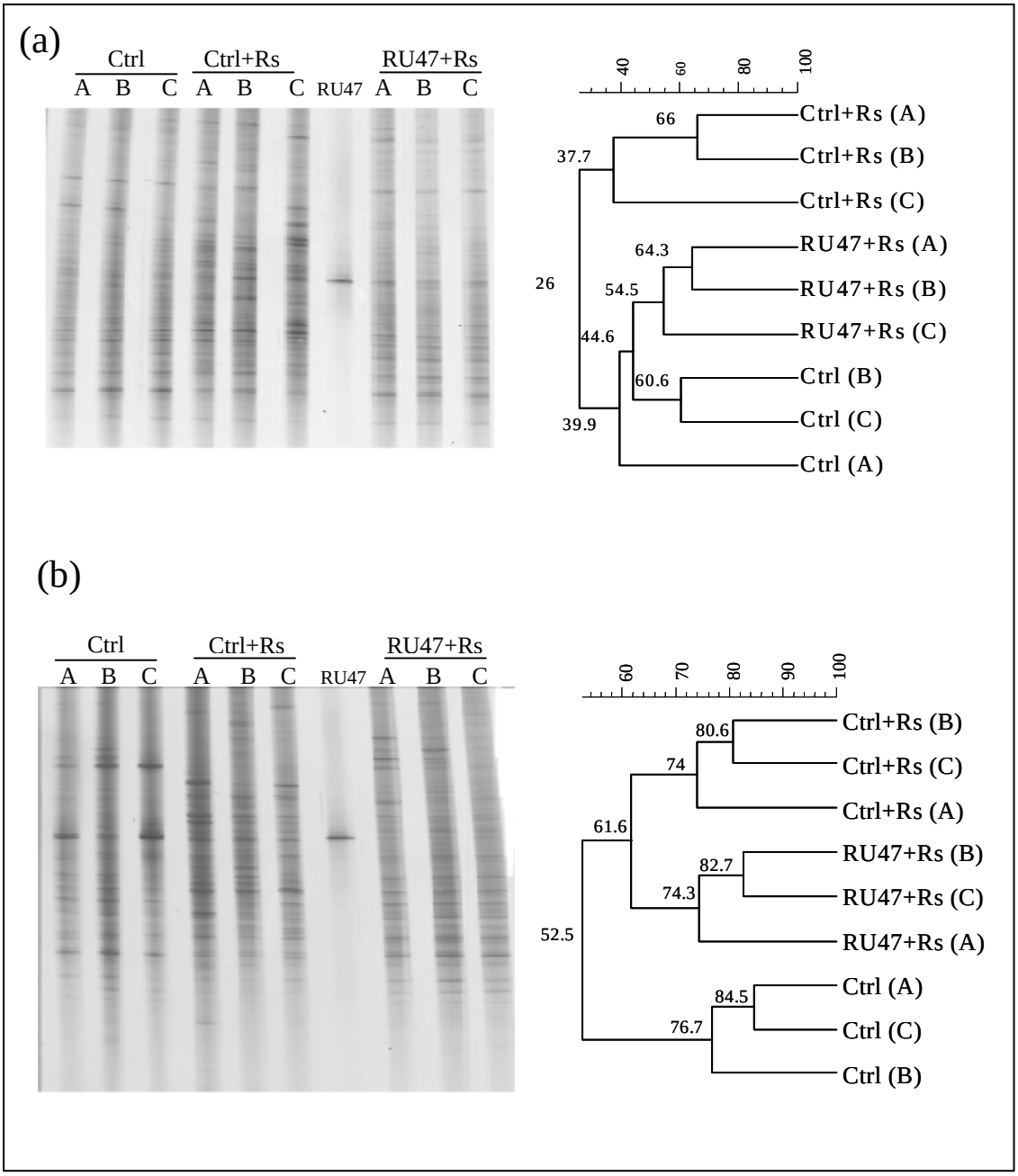

Figure 5.3. Comparison among DGGE fingerprints of bacterial 16S rRNA gene fragments amplified from community DNA extracts obtained from rhizosphere of lettuce plants without inoculation (Ctrl), with *R. solani* inoculation alone (Ctrl+Rs), and with combined inoculation of RU47 and *R. solani* (RU47+Rs) at (a) 5 WAS and (b) 7 WAS. Beside each gel is the corresponding dendrogram.

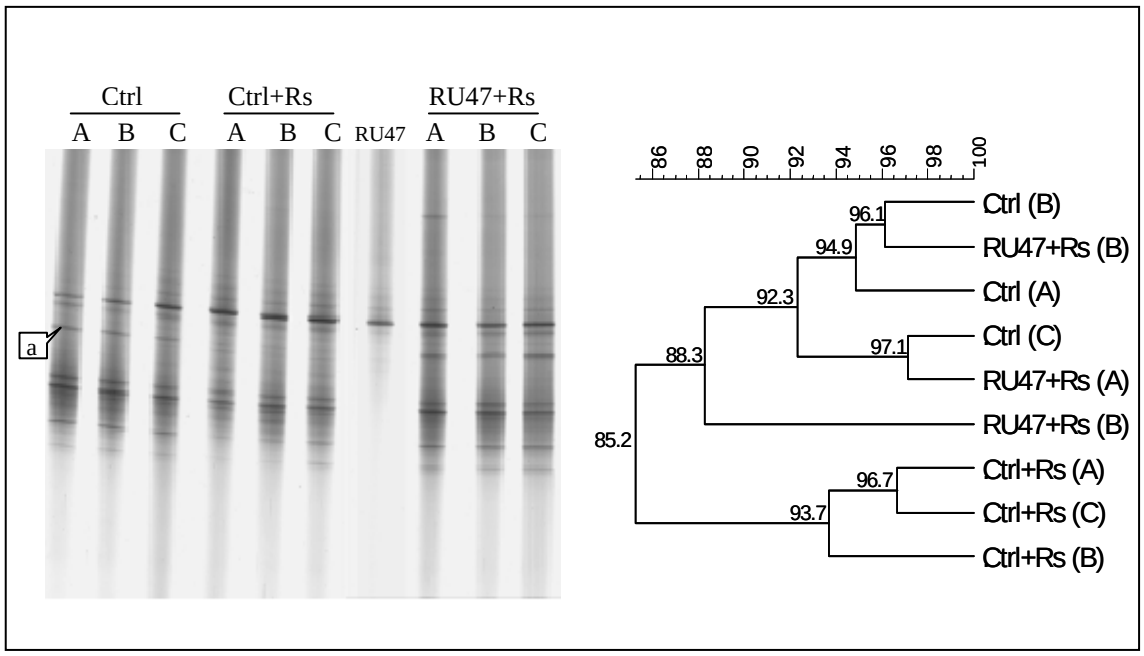

Figure 5.4. Comparison among DGGE fingerprints of *Pseudomonas*-specific 16S rRNA gene fragments amplified from community DNA extracts obtained from rhizosphere of lettuce plants without inoculation (Ctrl), with *R. solani* inoculation alone (Ctrl+Rs), and with combined inoculation of RU47 and *R. solani* (RU47+Rs) at 7 WAS. Beside the gel is the corresponding dendrogram.

In addition, UPGMA analysis of the *Pseudomonas* DGGE banding patterns confirmed that replicates of each treatment, inoculated or non-inoculated, shared a high similarity of 81.8 – 85.2% at all sampling times. Overall, a rather low degree of variability was observed among treatments and the genotypic diversity of this group was almost not affected by inoculation with RU47.

In contrast to the observation with the *Pseudomonas* 16S rRNA gene based community profiles, the DGGE profiles of the *Pseudomonas*-specific *gacA* gene fragments displayed a higher number of bands with better resolution. Furthermore, a band with the mobility of RU47 was detected only in the treatments with RU47 and this band was clearly separated from a dominant band, which was found in all other treatments (Figure 5.5a and 5.5b). Even at 7 WAS this dominant band migrated

closely to RU47 but did not possess the same position with RU47 isolate (Figure 5.5b) as seen in the DGGE band pattern of *Pseudomonas* community. Similarities between different treatments were high, 80.9%, 84.9%, and 60.7% at 3, 5 and 7 WAS respectively (Figures presented only for five and seven WAS, Figure 5.5a and 5.5b). Replicates from each treatment formed separate clusters at 3 WAS (data not shown) and 7 WAS, whereas all replicates of Ctrl, two replicates of RU47+Rs and one replicate of Ctrl+Rs formed a joint cluster at 5 WAS (two weeks after transplanting) (Figure 5.5a and 5.5b). The band patterns demonstrated that the genotypic composition of *Pseudomonas* carrying the *gacA* gene in lettuce rhizosphere was more diverse than the 16S rRNA gene based *Pseudomonas* community fingerprints, as more bands and a lower similarity among treatments were observed.

The DGGE profiles of fungal community revealed that a band with electrophoretic mobility corresponding to that of the 18S rRNA gene fragment amplified from *R. solani* isolate was much stronger in Ctrl+Rs treatment, whereas it was weak in RU47+Rs and Ctrl treatments (Figure 5.6a and 5.6b); indicating that the relative abundance of *R. solani* was increased in the roots of lettuce plants inoculated with *R. solani* alone while it was reduced in the RU47 inoculated plants. A high degree of variability between replicates of the same treatment and also between treatments inoculated with *R. solani* or not was observed. No cluster was obtained for the replicates of the healthy control or RU47+Rs treatments at 7 WAS (Figure 5.6b). A rather low degree of similarities was observed among treatments at 5 WAS and 7 WAS. Four dominant bands (Figure 5.6b, bands a,b,c,d) were found in Ctrl and RU47+Rs treatments which were weak or not detected in samples from Ctrl+Rs treatments at 7 WAS.

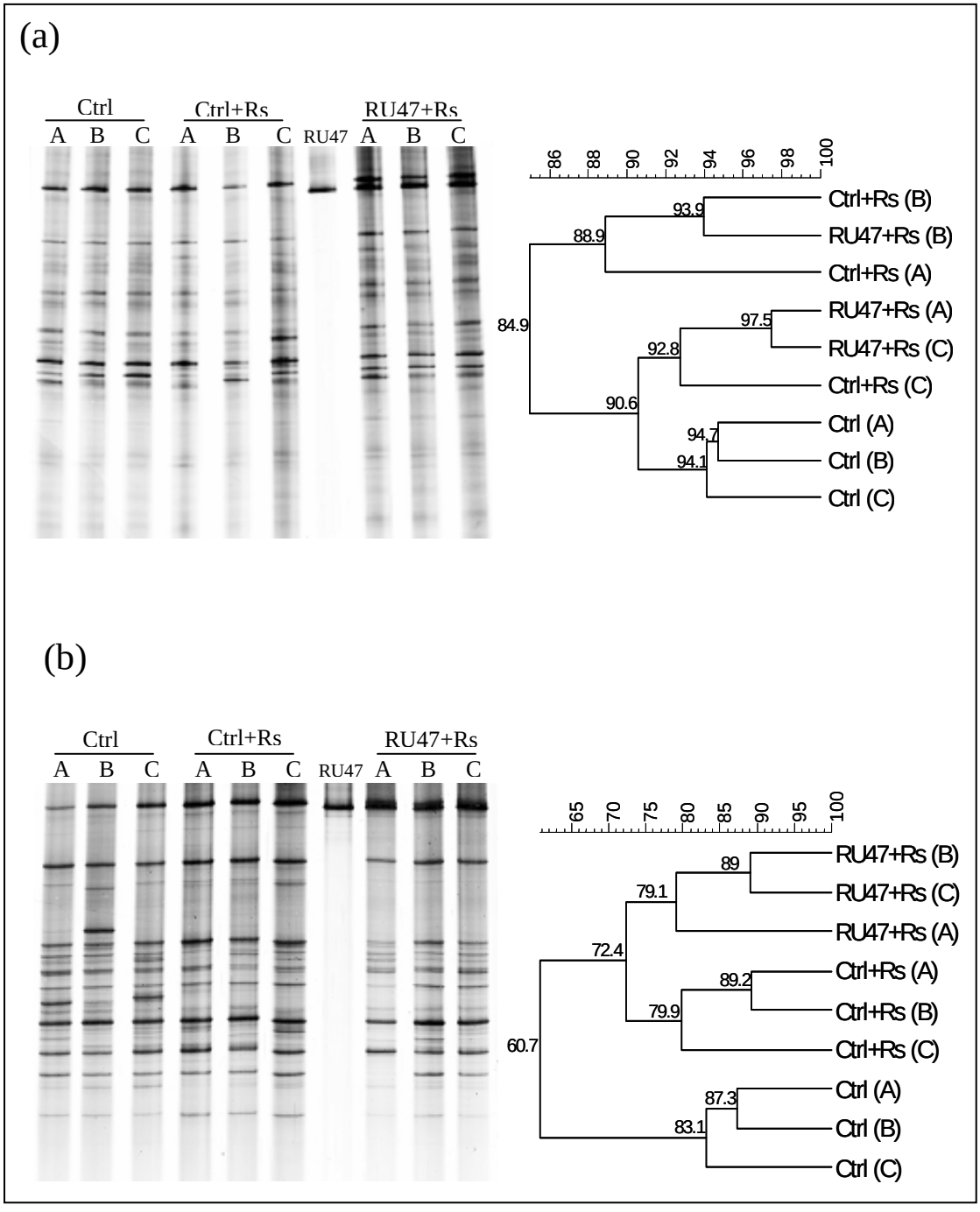

Figure 5.5. Comparison among DGGE fingerprints of Pseudomonas-specific *gacA* gene fragments amplified from community DNA extracts obtained from rhizosphere of lettuce plants without inoculation (Ctrl), with *R. solani* inoculation alone (Ctrl+Rs), and with combined inoculation of RU47 and *R. solani* (RU47+Rs) at (a) 5 WAS and (b) 7 WAS. Beside each gel is the corresponding dendrogram

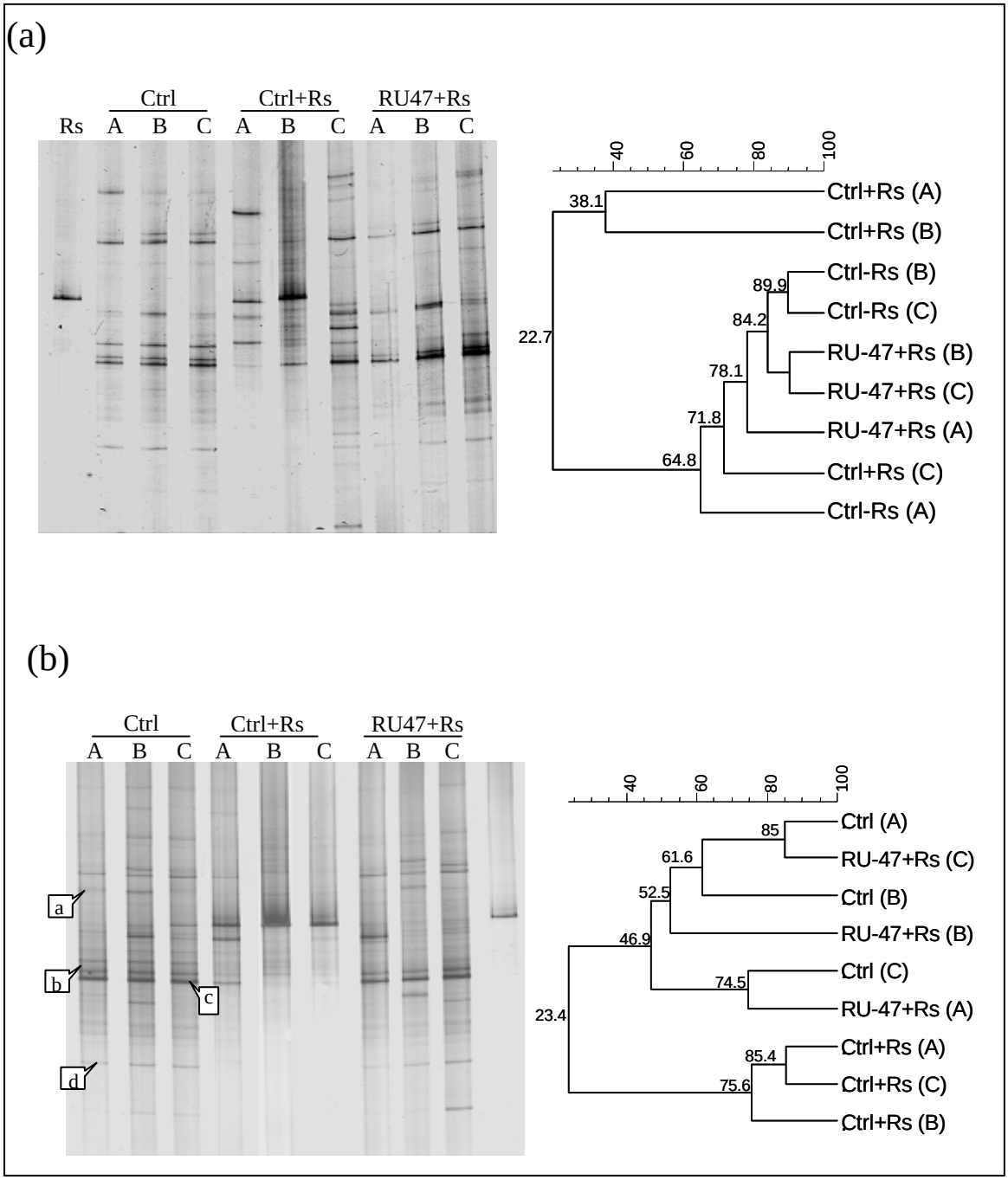

Figure 5.6. Comparison among DGGE fingerprints of fungal 18S rRNA gene fragments amplified from community DNA extracts obtained from rhizosphere of lettuce plants without inoculation (Ctrl), with *R. solani* inoculation alone (Ctrl+Rs), and with combined inoculation of RU47 and *R. solani* (RU47+Rs) at (a) 5 WAS and (b) 7 WAS. Beside each gel is the corresponding dendrogram.

3. Discussion

In this study ten bacterial isolates originating from four different suppressive soils (Adesina et al., 2007) were tested in growth chamber experiments with lettuce and *R. solani* AG1-IB in order to assess their potential to colonize lettuce roots, to suppress *R. solani* AG1-IB based disease symptoms and to cause changes in the relative abundance of the indigenous bacterial and fungal communities. The strains were selected from a collection of 327 antagonists (Adesina et al., 2007) based on their *in vitro* antagonistic activity against the potato pathogen *R. solani* AG3 and the lettuce pathogen AG1-IB and their ability to colonize lettuce seeds in a pre-screening greenhouse experiment (except for the *Streptomyces* isolate AN2-31). Seven of the ten strains selected were assigned by 16S rRNA gene sequencing to *Pseudomonas* and four of these strains carried the gene involved in 2,4-DAPG biosynthesis (*phlD)*. All potential 2,4-DAPG producing strains (KF36, KS16, KS74, KS90) were identified as *Pseudomonas fluorescens* by 16S rRNA gene sequencing (Adesina et al., 2007) and displayed strong *in vitro* antagonistic activity towards *R. solani* AG1-IB. The other *Pseudomonas* strains were identified as *P. fulgida* (KS70), *P. cannabina* (RN86) and *P. jessenii* (RU47). All *Pseudomonas* strains had in common that they displayed *in vitro* protease activity and produced siderophores. Furthermore, isolate AFNS31 showing high sequence identity with *Serratia marcescens,* isolate KF-32b with 99% identity with *Alcaligenes faecalis* and strain AN2-31 with 98% sequence identity with *Streptomyces purpureus* were tested in the growth chamber experiment. The only isolate that showed *in vitro* chitinase activity on plates was the *Serratia marcescens* strain AFNS31. All strains except for *P. jessenii* RU47 and *Serratia marcescens* strain AFNS31 displayed strong *in vitro* antagonistic activity towards *R. solani* AG1-IB (the pathogen of the model plant used for the growth chamber experiments).

In the growth chamber experiments, root and/or seed inoculation of the different

inoculant strains followed the common agricultural practice, transplating of the young lettuce plants three weeks after sowing. One day after transplating the plants were drenched with a cell suspension of the respective inoculants and *R. solani* AG1-IB was inoculated on barley kernels 24 hours later. Five weeks after sowing samples could only be taken from seven of the ten different treatments in experiment 1 because no plant survived in the presence of *R. solani* AG1-IB for the other three treatments. Similar observation was found in experiment 3 also for three of the treatments. In all, six out of ten *in vitro* antagonists of *R. solani* AG1-IB selected in our study failed to control the pathogen in the growth chamber assay. Three of these six isolates showed strong *in vitro* antagonistic activity against *R. solani* AG1-IB. Among the four isolates (RU47, KF36, KS16, and KS74) which suppressed the pathogen in the growth chamber experiment 1, only RU47 displayed efficient and consistent disease suppression throughout the four experiments. Interestingly, RU47 only had a weak inhibitory activity against *R. solani* AG1-1B *in vitro*. These findings confirm previous results that *in vitro* inhibition is one mechanism for biological control, but there is not necessarily a correlation between levels of *in vitro* inhibition and *in vivo* performance (Milus and Rothrock, 1997; Schottel et al., 2001; Faltin et al., 2004; Gravel et al., 2005).

The cfu counts determined three weeks after sowing in the growth chamber experiments were much higher than the counts obtained in the pre-screening greenhouse experiment. One reason for this difference might be that the soil composition was different. The ratio of sand to substrate of the soil used for greenhouse pre-screening experiment was 80:20 in contrast to the soil used for the growth chamber experiments where the ratio was 50:50. This observation might point to an influence of the soil on the ability of the inoculant strains to colonize the rhizosphere of lettuce. The ability of inoculant strains to colonize the root system after

seed inoculation or root inoculation has been pinpointed as a key factor for successful biocontrol by many authors (Bloemberg and Lugtenberg, 2001; Haas and Defago, 2005). Except for the *Streptomyces* strain AN2-31, all strains showed rather good seed colonization as evident by establishment of most inoculants at cell densities above 5×10^6 g^{-1} root fresh weight after three weeks of sowing. The poor colonization efficiency of the *Streptomyces strain* obviously have contributed to its failure to control the pathogen in the growth chamber experiment, despite its strong *in vitro* antagonistic activity towards a range of fungi and bacteria, including *R. solani* AG1-IB. However, in our study we also found strains, which failed to suppress bottom rot of *R. solani* but colonized lettuce roots at the same or similar population densities as the best isolate (RU47). This implies that inability of these isolates to suppress disease was not due to insufficient root colonization but may results from insufficient production of antifungal metabolites or the colonization of the strains at different locations than the infectious site of the pathogen or insufficient cell density of the antagonists at the infectious site. However, since the colonization patterns were not investigated in this study, it cannot be excluded that differences in colonization patterns between the strains existed. Using *gfp*-tagged inoculant strains several studies reported heterogeneous colonization patterns and the question was raised what triggers the colonization of some sites and not of others (Gamalero et al., 2003; Götz et al., 2006).

Another concern associated with the introduction of BCAs as inoculants is the possible influence on microbial community structure and processes that are essential to general soil ecosystem functioning (Winding et al., 2004). Plant inoculation with bacterial antagonists has been reported to influence microbial community structure as determined by culture-independent methods that rely on amplification of ribosomal RNA gene fragments from rhizosphere DNA extracts such as PCR-based denaturing

gradient gel electrophoresis (DGGE) (Götz et al., 2006), while for some studies, no significant observations were detected (Lottmann et al., 2000). In the present study, with PCR-DGGE analysis, the detection of RU47 was hampered in the bacterial community pattern due to the complexity of this pattern and the presence of ribotypes, which have similar electrophoretic mobility as RU47. However, the complexity of the DGGE pattern was reduced with the use of group-specific primers, thus, enhancement of the sensitivity of DGGE techniques. It was then obvious that RU47 belonged to the dominant ribotypes of 16S-based *Pseudomonas* and *gacA*-based *Pseudomonas*, as a band which corresponded in electrophorectic mobility to RU47 was found only in treatment inoculated with RU47 in the 16S-based *Pseudomonas* and *gacA*-based *Pseudomonas* community patterns. Furthermore, we observed that inoculation with RU47 had almost no effect on these two specific groups. This was demonstrated by a higher level of similarities obtained among treatments at each sampling time in comparison to lower degree of similarities found in the bacterial DGGE fingerprints. This observation contradicts the hypothesis that the organisms that are closely related to the BCA itself are most likely to be affected by the BCA due to competition for the same niches and resources (Winding et al., 2004). Moreover, the band patterns of the *gacA*-based *Pseudomonas* community revealed remarkable diversity of the *gacA* gene in the rhizosphere of lettuce plant (RU47-inoculated or non-inoculated) which would not have been detectable had only the 16S-based *Pseudomonas* patterns been analyzed. As found in this study, better resolution of *gacA*-based *Pseudomonas* community fingerprints than the 16S-based *Pseudomonas* community fingerprints was also reported by Costa et al. (2007). Our results also demonstrated that inoculation with RU47 and/or *R. solani* had more impacts on the fungal community structure than the bacterial community structure (universal or group-specific). In particular, a considerable decrease in the relative

abundance of the pathogen in the rhizosphere of lettuce inoculated with RU47 was found. In addition, we found a high variability among replicates of the same treatments, which may be due to uneven distribution of fungi or the pathogen mycelia in the rhizosphere. The variability may also be due to inability to quantify the amount of pathogen inocula used per plants for inoculation.

In conclusion, out of the ten *in vitro* antagonists investigated in this study, we found only one promising strain, *Pseudomonas jessenii* RU47. For this strain, *in vitro* proteolytic activity and siderophores production were displayed, while chitinolytic, glucanolytic and cellulolytic activities, and genes encoding antibiotics such as 2,4-diacetylphloroglucinol, phenazine, pyrrolnitrin, pyoluteorin were not detected. HPLC analysis of the culture filtrates of RU47 detected a small size peptide; however, no known anti-fungal metabolite was found (data not shown). *In vitro* production of salicylic acid as siderophores by several resistance-inducing bacteria at low-iron conditions has been reported and its role in induce-systemic resistance (ISR) elicitation process was demonstrated in the case of *P. aeruginosa* KMPCH (de Meyer et al., 1999). Hence, siderophore production has been suggested to trigger induce systemic resistance (ISR) signal pathway in plants (Audenaert et al., 2002; van Loon and Bakker, 2005). Considering the strong and consistent suppression of the pathogen by strain RU47 in the presence of the host plant *in vivo*, which is contrary to the weak and rather insignificant direct inhibition of *R. solani* AG1-IB by this strain *in vitro,* couple with the *in vitro* production of siderophores by strain RU47. Thus it appears that induction of systemic resistance in lettuce is the possible mechanism by which strain RU47 could protect lettuce plant against *R. solani* AG1. Nonetheless, its exact mechanism still remains unclear and its explanation needs future investigation. Strain RU47 did not promote shoot nor root biomass of lettuce compared to the non-inoculated healthy plant in the absence of the pathogen. On the basis of this

observation we suggest that for biological application under the condition in which the strain was tested, it can only be used to control bottom rot disease in a soil infested with the pathogen and not for growth promoting purpose under non-infested conditions.

4. Experimental procedures

4.1 The microorganisms: origin, cultivation and maintenance

Bacterial antagonists used in this study were isolated from four soils with documentation of natural suppression to soil-borne pathogens (Adesina et al., 2007). The origin of the suppressive soils and the suppressed phytopathogens (in parentheses) are as follows: the United Kingdom (*Gaeumanomyces graminis),* the Netherlands (*Rhizoctonia solani* AG3*)*, Sweden (*Plasmodiophora brassicae)* and France (*Fusarium oxysporum f.sp lini).*

Virulent strains of *Rhizoctonia solani* AG1-IB and AG2 were provided by the laboratory of Dr. Rita Grosch (Institute for Vegetables and Ornamental Crops, Großbeeren, Germany). Fungal strains were maintained on Waksman agar (WA) containing 5 g of proteose-peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck, Darmstadt, Germany), 3 g of meat extract (Chemex, München, Germany), 5 g of NaCl (Merck, Darmstadt, Germany), 20 g of agar (Difco, Detroit, MI, USA), and distilled water (to 1 liter) (pH 6.8). Bacterial strains were maintained on R2A agar (Difco, Detroit, MI, USA).

4.2 Screening of *in vitro* antagonists of *R. solani* AG3 for activity against two other anastomosis groups of *R. solani* (AG2 and AG1-IB)

Approximately 15 out of 248 antagonists isolated from the four suppressive soils were selected on the basis of their (i) strong *in vitro* activity against *R. solani* AG3 or dual activity towards *R. solani* AG3 and *F. oxysporum* Foln3. These isolates were further tested for inhibitory activity against two other anastomosis groups of *R. solani* (AG1-IB and AG2). Assessment of the *in vitro* activity against *R. solani* AG1, was necessary because this anastomosis group is the pathogen of the model plant (lettuce) selected for the growth chamber experiments. *In vitro* dual-culture inhibitory activity against *R. solani* AG1-IB and AG2 was determined as described by Adesina et al. (2007).

4.3 Generation of antibiotics resistant mutants

To facilitate re-isolation of the bacterial inoculants from the rhizosphere, spontaneous rifampicin resistance mutants of bacterial antagonists were generated by plating overnight culture on R2A agar supplemented with 75 µg ml^{-1} rifampicin. To ascertain the antagonistic activity of the antibiotics-resistant *strains,* they were again tested *for in vitro* inhibitory activity of *R. solani* (AG1 and AG2). The mutant strains were stored at –80^{o} C in Luria-Bertani (LB) broth (ROTH, Germany) containing 20% glycerol supplemented with rifampicin (75 µg ml^{-1}).

4.4 Pre-screening experiments in the greenhouse

To determine the ability of the mutant strains to colonize the rhizosphere of the selected model plant (lettuce), a pre-screening experiment was carried out in the greenhouse. The lettuce seeds were surface-sterilized in 2% sodium hypochlorite (NaOCl) for 5 mins followed by washing steps in sterile distilled water for three times.

The seeds were pre-germinated for two days at room temperature in sterile petri dishes underlaid with sterile moistened filter papers. Pre-germinated lettuce seeds were soaked in cell suspension (cell concentration corresponding to approximately 10^9 cells ml^{-1}) of each antagonist for one hour. Four pre-germinated seedlings per pot were sown into non-sterile potting soil [turf substrate/clay granulate No. 4230, Klasmann-Deilmann GmbH, Geeste, Germany, sieved (2mm mesh width) and mixed with 80% (w/w) sand]. The pots were kept in the greenhouse at 20^{o}C, 30% humidity and daylight. Each treatment was replicated three times. Rhizosphere samples were collected after four weeks of planting and processed mechanically by Stomacher blending for 3 min at high speed. Serial dilutions of the rhizosphere suspensions were plated on R2A supplemented with rifampicin (75 µg ml^{-1}) and cycloheximide (100 µg ml^{-}) and the colony forming units (cfu) were determined after two days of incubation at 28^{o}C.

4.5 Growth chamber experiments

Nine antagonistic isolates with ability to colonize lettuce roots at cell densities of approximately $\log_{10}$ 4 or more were selected for the growth chamber experiments. In addition, one *Streptomyces* isolate which had shown multi-target activity against different fungi and bacteria *in vitro* was selected; but the root colonization efficiency of this strain in the greenhouse was not determined due to initial inhibition of *Streptomyces* growth on rifampicin amended agar plates. However, the antibiotic mutant of this isolate was finally generated using combination of chloramphenicol (20 µg ml^{-1}), nalidixic acid (15 µg ml^{-1}) and sulfadiazine (20 µg ml^{-1}). Efficacy of these ten antagonistic isolates in controlling bottom rot disease caused by *R. solani* AG1-IB on lettuce plants cv. Tizian was assessed under favourable conditions for the

pathogen in the growth chamber (York, Mannheim, Germany, 20/15 ℃, 60/80% relative humidity, 16/8 hour day/night cycle, 500 µmol m^{-2} s^{-1}). The experiment was repeated four times. In experiment 1, all the ten *in vitro* antagonists were evaluated. Four isolates with the best disease suppression were repeated in experiments 2 and 3, while only two isolates with the best results in experiments 1 and 2, were assessed in experiment 4. In experiments 3 and 4, growth-promoting effects of the antagonists on shoot and root dry mass of lettuce plants was investigated in treatments with only bacterial inoculation (without pathogen inoculation).

4.6 Seed bacterization and plant treatment with antibiotic-resistant strains

Each antagonist (except *Streptomyces*) was grown overnight on nutrient agar supplemented with rifampicin at 75 µg ml^{-1} while *Streptomyces* was grown for three days on the same agar supplemented with chloramphenicol at 20 µg ml^{-1}, nalidixic acid at 15 µg ml^{-1} and sulfadiazine at 20 µg ml^{-1}. The overnight or three days cell culture were scooped, washed and suspended in sterile saline (0.3% NaCl) followed by shaking in order to render a turbid suspension. The concentration was adjusted in a spectrophotometer, to optical density corresponding approximately to 10^9 cfu ml^{-1}. Lettuce seeds cv. “Tizian” were soaked in the bacterial suspension for one hour at room temperature. Seeds that were incubated in sterile saline for the same time served as control. Seeds were germinated in a seedling tray containing 92 holes filled with non-sterile mixture of sand and substrate (Fruhsdorfer Einheitserde Typ P, Germany) at 1:1 ratio (v/v). For each bacterial inoculated treatment, ten seeds removed from the sand-substrate mixture after 10 min of sowing were used for determination of the initial inoculum densities on seeds. The seeds were vortexed vigorously for one minute in glass tubes containing 2 ml of sterile saline and plated

after serial dilution on R2A agar supplemented with the corresponding antibiotics and cycloheximide (100 µg ml^{-1}). Colony forming units (cfu) per seed were determined after three days of inoculation at 28^{o}C.

At 2-3 leaf stage (approximately three weeks after planting), lettuce seedlings were transplanted into pots (one plant per pot) filled with non-sterile mixture of sand and substrate (Fruhsdorfer Einheitserde Typ P, Germany) at 1:1 ratio (v/v). Each treatment consisted of five plants with six replications in a randomized design. Plant treatment with suspension of antagonistic bacteria followed 24 hours after transplanting. Briefly, each antagonistic isolate was grown overnight (*Streptomyces* isolate was grown for three days) at 28° C in nutri ent broth supplemented with the corresponding antibiotics. The bacterial cells were pelleted and washed by centrifugation at 13,000 x *g* for 5 min, and suspended in sterile saline. The cell suspension was adjusted to an optical density corresponding to a cell density of approximately 10^{7} cfu ml^{-1}. Each plant received 20 ml of the bacterial suspension. Non-inoculated treatments, which serve as control, received 20 ml of sterile saline.

4.7 Preparation of *R. solani* AG1-IB inoculum

Sterile barley kernels infested with *R. solani* AG1-IB (strain RS 7/3) for three weeks in the dark were used as pathogen inoculum on lettuce (Schneider et al., 1997). The healthy control treatment (Ctrl) consisted of sterilised non-infested barley kernels. A day after plant treatment with suspension of bacterial antagonists, the kernels were placed 1 cm deep at a distance of 2 cm from each plant. Each plant received five infested or non-infested barley kernels. The pots were watered lightly to maintain the substrate moisture.

4.8 Suppression of bottom rot disease of *R. solani* AG1-IB by bacterial inoculants

Seven weeks after sowing (four weeks after pathogen inoculation), which marked the end of each experiment, a total of 24 plants was evaluated for (i) suppression of bottom rot disease of *R. solani* AG1-IB based on the number of surviving plants, (ii) disease severity based on the percentage reduction in shoot dry mass (SDM). The shoot dry mass of the non-inoculated healthy control (without pathogen or bacteria inoculation) (Ctrl) was set to 100% to represent the expected shoot dry mass in healthy non-inoculated plants. Percentage reduction in SDM for other treatments was calculated in relation to the Ctrl.

4.9 Root colonization by bacterial inoculants

To determine the survival and root colonization efficiency of the inoculated antagonists, rhizosphere samples were collected at three time points (3, 5, and 7 weeks after sowing, WAS). For each treatment and sampling time three replicates were used. Loosely adhering soils were removed from the roots by shaking. Five grams of roots were suspended in 20 ml of sterile 0.3% NaCl and shaken vigorously in sterile glass flask containing 6 glass beads (0.6 mm in diameter) on a rotary shaker for 1 hour at 307 rpm. Aliquots of the rhizosphere suspension were immediately processed for (i) enumeration of the introduced strains on selective agar plates, (ii) centrifuged for 5 min at 13,000 x g and the cell pellets kept at –20^{o}C for DNA extraction. For enumeration of the introduced strains, rhizosphere suspensions were serially diluted and plated on R2A medium (Difco, Detroit, MI, USA), supplemented with chloramphenicol (20 µg ml^{-1}), nalidixic acid (15 µg ml^{-1}) and sulfadiazine (20 µg

ml^{-1}) for treatment inoculated with *Streptomyces* and rifampicin (75 µg ml^{-1}) for other antagonists. Cycloheximide was added to all media (100 µg ml^{-1}).

The ability of the most promising antagonist to colonize the vascular tissue of lettuce roots and leaves was determined at 7 WAS in experiment 3. Roots from the plant inoculated with the antagonist were shaken to remove loosely adhering soils and washed in running water. One gram of the washed roots or leaves were soaked in 1% sodium hypochlorite for one min. Surface-sterilized root or leaf samples were rinsed four times with sterile distilled water and macerated using sterile mortal and pestle. The suspensions of the macerated roots or leaves were serially diluted and plated as mentioned above.

4.10 DNA extraction from rhizosphere samples

Cell pellets from the rhizosphere suspension collected at each time point were processed for DNA extraction with Bio101 extraction kit (Q.BIOgene, Carlsbad, CA) according to the protocol supplied by the manufacturer. After DNA extraction, final DNA purification was performed using the GENECLEAN Spin kit (Q.BIOgene, Carlsbad, CA). DNA yields were checked after electrophoresis of sub-samples in 1% agarose gels stained with ethidium bromide under UV light. DNA was quantified visually by comparison to a 1-kb gene-rulerTM DNA ladder (Fermentas, St. Leon-Rot, Germany) applied on the agarose gels. Genomic DNA samples were diluted differentially to obtain approximately 1 to 5 ng DNA to be used as PCR-templates for the bacterial taxa, while c. 20 ng DNA was used as a template for the fungi.

4.11 PCR amplification of bacterial and group-specific 16S rRNA gene fragments for DGGE analysis

To amplify the 16S rRNA gene fragments of *Pseudomonas* from rhizosphere DNA extracts, a nested PCR system was used. For the first PCR, the primer system F311Ps/R1459Ps and PCR conditions described by Milling et al. (2004) were used (Table 5.5). Diluted amplicons obtained from the first PCR served as templates for a second PCR using the bacterial primers F984-GC/ R1378 (Table 5.5) and PCR conditions described by Heuer et al. (1997). To generate universal bacterial community fingerprints the primer system from the second PCR was used directly to amplify 16S rRNA gene fragments from rhizosphere DNA extracts.

4.12 PCR amplification of *gacA* gene fragments

Amplification of the *Pseudomonas* specific *gacA* gene fragment (575 size in bp) was carried out using a nested PCR approach. The first PCR was carried out with the primers gacA-1F and gacA2, diluted amplicons obtained from the first PCR served as templates in a second nested PCR step with the primers gacA-1FGC and gacA-2R (de Souza et al., 2003c; Costa et al., 2007) (Table 5.5). PCR conditions described recently by Costa et al. (2007) were used.

4.13 PCR amplification of fungal-specific 18S rRNA gene fragments

Amplification of 18S rRNA gene fragments (1,650 bp) was done using the primer pair NS0/EF3 (Table 5.5) in a first PCR assay followed by a second PCR step with the primer pair NS1/FR1GC (Table 5.5). The PCR conditions used were described recently by Costa et al. (2006b).

4.14 Denaturing gradient gel analysis (DGGE)

DGGE analysis was performed with the Dcode System apparatus (Bio-Rad Inc., Hercules, CA). The polyacrylamide gel was a gradient gel containing 9% (fungi) or a double gradient gel of 6-9% (bacteria, *Pseudomonas* and GacA*)* acrylamide with a denaturant gradient of 18-38% (fungi) or 26-58% (bacteria, *Pseudomonas* and GacA) of denaturant according to Gomes et al. (2004) (where 100% denaturants contain 7M urea and 40% formamide). Aliquots of PCR samples (2–4 ul) were applied to the DGGE gels, and the run was performed in 1X Tris-acetate-EDTA buffer at 58℃ with a constant voltage of 220 V for 6 h (b acteria and *Pseudomonas* and GacA) or 180 V for 18 h (fungi). The DGGE gels were silver-stained, according to Heuer et al. (2001).

4.15 Cluster analysis of DGGE fingerprints

Fungal, bacterial, 16S rRNA-based *Pseudomonas* and *gacA*-based *Pseudomonas* community fingerprints obtained from DGGE were analyzed with the software package Gelcompar II version 5.6 (Applied Maths, Kortrijk, Belgium). Background was subtracted by a rolling disk method with an intensity of 10 (relative units), and the lanes were normalized.

A dendrogram was constructed by the Pearson correlation index for each pair of lanes within a gel and cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA).

4.16 Statistical analysis

Data on bacterial densities in the rhizosphere of lettuce plants (cfu) was $\log_{10}$ transformed prior to statistical analysis) and plant shoot and root dry mass were analyzed after ANOVA using the Tukey's test procedure (HSD) with $P = 0.05$.

Table 5.5. Primer sequences and annealing temperatures used for PCR experiments in this study

Primer	Sequence 5`–3´	Annealing temperature	Target	Reference
F311Ps R1459Ps	CTGGTCTGAGAGGATGATCAGT/ AATCACTCCGTGGTAACCGT	63 ^{o}C	Pseudomonas Pseudomonades	Milling et al., 2004 Milling et al., 2004
R1378 F984 GC-Clamp	CGG TGT GTA CAA GGCCCGGGAACG AACGCGAAGAACCTTAC CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGG GGG G	57 ^{o}C	Bacteria Bacteria	Heuer et al., 1997 Heuer et al., 1997 Nübel et al., 1996
gacA-1F gacA2	TGATTAGGGTGYTAGTDGTCG MGYCARYTCVACRTCRCTGSTGAT	57 ^{o}C	*gacA* gene *gacA* gene	Costa et al., 2007 de Souza et al., 2003c
gacA-2R gacA-1F- GC-Clamp	GGTTTTCGGTGACAG GCA CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGGGATTAGGGTGCTAGTGGTCGA	52 ^{o}C	*gacA* gene *gacA* gene	Costa et al., 2007 Costa et al., 2007 Costa et al., 2007
NSO EF3	TACCTGGTTGATCCTGCC TCCTCTAAATGACCAAGTTTG	53 ^{o}C,	Fungi Fungi	Messner and Prillinger, 1995 Smit et al., 1999
NS1 FR1 GC-Clamp	GTAGTCATATGCTTGTCTC AICCATTCAATCGGTAIT CCC CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCCG	48 ^{o}C,	Fungi Fungi Fungi	White et al., 1990 Vainio & Hantula, 2000 Vainio & Hantula, 2000

CHAPTER 6

CHAPTER 6

General discussion and conclusions

Over the last decades, efforts have been directed towards developing new alternatives to chemical disease control, due to the deleterious effect of chemical pesticides on the environments and human health (Mark et al., 2006). Many studies have reported on plant-beneficial fungi and bacteria with natural activity against plant pathogens, and this is considered as a very appealing and ecologically friendly alternative to the use of chemical fungicides. In some soils, the population and activity of one or several specific groups of such plant-beneficial microorganisms are very significant to the extent that the soils become inhospitable to some specific plant pathogens without the use of chemical pesticides, a term referred to as specific suppression or natural disease suppression (Weller et al., 2002). These naturally disease-suppressive soils are located all over the world, have been described for many soil-borne plant pathogens (Cook and Bakker 1983; Weller et al., 2002; Haas and Defago, 2005); and are regarded as sources of natural, effective and valuable plant-beneficial microorganisms for combating plant diseases (Weller, 1988; Haas and Defago, 2005). Such systems can serve potentially as models for understanding the mechanisms by which complex plant-associated microorganisms interfere with plant pathogenesis. As a result, during the past 20 years, many biocontrol organisms have been isolated from soils naturally suppressive to certain soil-borne plant pathogens (Larkin et al., 1996; Martunez et al., 2002). Most studies on the search for biocontrol agents have focused also on normal agricultural soils, roots, seeds and diverse environmental samples (Hoitink and Boehm, 1999; Cavaglieri et al., 2004; Islam and Toyota, 2004; Berg et al., 2005). Thus, several antagonistic bacteria and fungi have been isolated, but very few have made it up to the commercial level as

biopesticides and biofertilizers (Kiely et al., 2006). Therefore, there is need to search for more biocontrol agents in order to meet the increasing demand for pesticide-free agricultural produce. In light of this, the focus of the work presented in this thesis is directed towards characterizing bacteria with antagonistic activity towards *R. solani* and *F. oxysporum* from agricultural soils originating from different ecological zones in Europe. In addition, the composition and diversity of the bacterial antagonists in each soil were assessed, and selected strains were evaluated for their potential in biocontrol application. While Chapter 2 of the thesis focused on the short review on the existing knowledge or studies relating to the subjects of the research work, Chapters 3-5 summarize the research work carried out, the methodologies and the results obtained. Here, the summary on the research results, their implications and application for future use are discussed.

Chapter 3: Screening of bacterial isolates from various European soils for antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed

The objective of the study described in Chapter 3 of this thesis is to determine whether the proportion and taxomonic compositions of culturable bacteria with antagonistic activity towards *R. solani* and *F. oxysporum* differs among four soils with previous documentation of natural disease suppression located in France (FR), the Nethelands (NL), Sweden (SE) and the United Kingdom (UK), and two soils without documentation of disease suppression situated in two locations in Germany, Berlin (G-BR) and Braunschweig (G-BS). In addition, the study aimed to determine whether particular antagonistic attributes could be related to a given site, functioning as a potential indicator of major antagonistic mechanisms that confer suppressiveness to these soils.

Bacteria such as those belonging to the genera, *Pseudomonas* and *Streptomyces* among others, have been implicated in biological control of plant pathogens (Weller et al., 2002; Emmert 1999; Liu et al., 1996). Thus, the isolation procedure employed in this study focused on aerobic bacteria (which were retrieved on R2A medium), and the species of the bacterial genera *Streptomyces* and *Pseudomonas* (isolated on selective media, AGS and King´s B media respectively). Approximately equal numbers of bacteria were isolated on each medium per site and were screened *in vitro* for antagonistic activity against two fungal pathogens, *R. solani* AG3 and *F. oxysporum* f.sp. lini (foln3) in dual tests. Isolates with anti-fungal activity were also tested for their ability to inhibit bacteria as an indication of antibiotic production. *Ralstonia solanacearum* (a causal agent of bacterial wilt in many crops) was used as a model gram-negative bacterium while *Bacillus subtilis* was selected as gram-positive bacterium. Our results revealed the presence of bacteria with antagonistic activity towards the two pathogens in all soils, irrespective of previous documentation of disease suppression; which corroborates earlier observations that, virtually, all natural and agricultural soils possessed some ability to suppress the activity of soil-borne plant pathogens as a result of the presence and activity of soil microorganisms (Cook and Baker, 1983; Weller et al., 2002; Mazzola, 2004). Nonetheless, bacterial antagonists of *F. oxysporum* were higher for the four suppressive soils than the two soils without documentation of suppression. A higher proportion of isolates active towards *R. solani* found in the NL compared to other soils agrees with the natural suppressiveness of this soil towards *R. solani* (Garbeva et al., 2004). Moreover, more than half of the antagonists isolated from SE and the NL were active against the potato pathogen, *R. solanacearum*, suggesting, these soils may possess a degree of natural suppression towards this pathogen. However, further *in vivo* studies will be necessary to validate these findings. Testing representative collections of soil

bacteria for *in vitro* antagonistic activity indicated that the suppressive soils harbor a higher proportion of antagonists than the soil without previous documentation of disease suppression.

Identification by FAME and/or 16S rRNA gene sequencing revealed that taxonomic composition and diversity of bacteria with antagonistic activity differed for each soil. These differences may be due to their different cropping history, soil types and location. Plant species, soil type, land use history and management, among others, are the main factors governing the structure and composition of bacterial communities in any given soil (Garbeva et al., 2004; Costa et al., 2006b). Antagonist diversity was highest for the French soil (FR), where 14 different genera with *in vitro* antagonistic activity were isolated, of which seven genera were exclusively from this soil. While the highest proportion of antagonists in this soil were *Streptomyces* and *Stenotrophomonas*; Swedish (SE) and Dutch soil (NL) were dominated by *Pseudomonas* and *Streptomyces* antagonists. It is clear that the majority of the antagonists represented in our collection are *Pseudomonas* and *Streptomyces* spp., which reflects the strategy of selective isolation on KMB and AGS used in this study. Nevertheless, even on R2A these two genera together with *Bacillus* were the most frequently isolated antagonists found nearly in all soils, suggesting their dominance and antagonistic potential in these soils. Bacterial genera, such as *Pseudomonas, Bacillus* and *Streptomyces* were also found as dominant antagonists of *Verticillium dahliae* in the rhizosphere of oilseed rape and strawberry, and the bulk soil (Berg et al., 2006). The biocontrol potential of these bacterial groups has been repeatedly demonstrated in several studies (Liu et al., 1996; Emmert and Handelsman, 1999; Weller et al., 2002). Our collection of antagonists also contained less frequently reported bacterial antagonists such as the genera *Dyella*, *Ochrobactrum*, *Brevibacillus* and *Variovorax*, which biocontrol activity has received less attention.

Microbial antagonists are able to interfere with pathogen growth, and survival through several mechanisms, including competition for nutrients (e.g iron via production of siderophores); or parasitism that may involve production of cell-wall degrading enzymes such as glucanase, protease, cellulase and chitinase; or inhibition of pathogen by antibiotics production (Fravel, 1988; Raaijmakers et al., 1997; Lugtenberg et al., 2001; Berg et al., 2005; Kobayashi et al., 2005; Dahiya, 2005). Understanding the mechanisms of activity of antagonistic bacteria is necessary for successful biotechnology application. Therefore, the collection of bacterial antagonists retrieved in this study, were phenotypically characterized *in vitro* for possible mechanisms of antagonistic activity based on glucanase, cellulase, protease, chitinase and siderophore production. Our results revealed that the production of siderophore and protease were most prominent traits among the antagonists. Nonetheless, no correlation between antagonism of a specific fungus and the tested phenotypic traits (enzyme and siderophore productions) for the different soils was found. However, a positive correlation between *in vitro* antagonistic activity towards *R. solanacearum* and siderophore production among *Pseudomonas* antagonists was found.

Chapter 4: Diversity of *Pseudomonas* specific-*gacA* gene among culturable antagonistic *Pseudomonas* isolates and in the bulk soils using PCR-DGGE analysis, and detection of antibiotic producing genes.

The bacterial genus, *Pseudomonas*, has been frequently reported as successful biocontrol agents. Indeed, few *Pseudomonas* biocontrol strains have been developed recently in the USA for commercial purpose (Mark et al., 2006). *Pseudomonas* isolates represented the second to the largest group of bacterial antagonists retrieved in this study and they were the focus of the study presented in

Chapter 4 of this thesis. The antibiotics produced by numerous strains of *Pseudomonas* with antagonists activity, have been demonstrated to play a major role in biological control of plant pathogens (Chin-A-Woeng et al., 2001; Raaijmakers et al., 2002; Haas and Defago, 2005). Such antibiotics include 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine (Phz), pyrrolnitrin (PRN) and pyoluteorin (PLT). Identification of gene clusters responsible for the production of these antibiotics has facilitated the design of primers and the use of polymerase chain reaction (PCR) methods to rapidly detect the antibiotic genes, making their detection in producing organisms possible (Raaijmakers et al., 1997; de Souza and Raaijmakers, 2003).

Characterization of the *Pseudomonas* antagonists isolated in this study based on the detection of the genes involved in the production of the aforementioned antibiotics by PCR-Southern blot hybridization revealed that *Pseudomonas* antagonists containing 2,4-DAPG encoding gene (*phlD*) were detected in all soils. *Pseudomonas* antagonists carrying the gene involved in production of PRN were scarcely represented. Antagonists isolated in this study contained neither the *phzCD* nor the *pltC* genes. The detection of *Pseudomonas* antagonists carrying the gene encoding 2,4-DAPG in all the six European soils studied, ties in well with the reports of Keel et al. (1996) and Raaijmakers et al. (1997) that 2,4-DAPG-producing *Pseudomonas* spp. occur in diverse soils of worldwide origin. *Pseudomonas* carrying the 2,4-DAPG encoding gene were found in all soils at varying proportions, the frequency was highest for Swedish soils, where approximately 50% of 38 *Pseudomonas* antagonists retrieved from this soil contained the *phlD* gene. This fraction represented 59% of the *phlD* positive antagonists obtained from all the six sites. A far higher proportion of *phlD*$^+$ antagonists found in Swedish soil than in other soils, suggests that 2,4-DAPG producing *Pseudomonas* antagonists might belong to a significant fraction of

Pseudomonas population in Swedish soil and they may likely play an important role in the natural disease suppression of this soil. *Pseudomonas* spp. that produce the antibiotic 2,4-DAPG inhibit several plant pathogens (Raaijmakers et al., 1997; Weller et al., 2002); their dominance have been positively correlated with natural disease suppression of take-all decline in Dutch soil, *Fusarium* wilt in US, and *Thielaviopsis* root rot of tobacco in Swiss soil (Weller et al., 2002; Haas and Defago, 2005).

Key factors in the regulation of the biosynthesis of several fungal metabolites, including those investigated in this study (antibiotics and extracellular protease) are global regulation and quorum sensing. A two-component system, GacS/GacA, is an example of a well-studied global regulatory system that controls the expression of genes involved in biocontrol traits of many plant-beneficial gram-negative bacteria (Heeb and Haas, 2001). Mutations in *gacA* or *gacS* have been demonstrated to have diverse negative effects on the expression of various genes involved in bioysnthesis of secondary metabolites essential for biocontrol activity (Laville et al., 1992; Heeb and Haas, 2001). This system has been particularly studied in *Pseudomonas* spp. In an *in silico* study, the GacA protein of *Pseudomonas* spp. was found to cluster separately from GacA homologues of enteric bacteria, *Xylella* and *Shewanella* spp., suggesting that the *gacA* gene may be conserved within the genus *Pseudomonas* (Heeb and Haas, 2001). Indeed, with the design of the primers targeting *gacA* gene in *Pseudomonas* spp., the gene was shown to be highly conserved within this genus (de Souza et al., 2003c). As a result, these authors proposed the use of *gacA* gene as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples. With the development of a novel PCR-DGGE system by Costa et al. (2007), the detection, composition and diversity of *Pseudomonas*-specific *gacA* gene, especially in a complex environment such as rhizosphere or bulk soil have been achieved. In Chapter 4, this novel PCR-DGGE system was used to

determine the diversity of *gacA* gene among our collection of *Pseudomonas* antagonists. They were grouped into different *gacA* genotypes according to the DGG electrophoretic mobility of the PCR-amplified *gacA* gene obtained from their genomic DNA. Our data showed that the *gacA* gene was highly diverse among the collection of *Pseudomonas* antagonists obtained from each site. The level of resolution allowed by the strain-specific BOX-PCR fingerprints obtained from the genomic DNA of *Pseudomonas* isolates was used to assess the differenciation provided with DGGE of *gacA* fragments. The number of genotypes detected by BOX-PCR was higher than *gacA* DGGE for antagonists obtained from all sites, except G-BR. Inspite of this, we still found a good correlation between the two methods for some of the antagonists. In particular were six antagonists from NL identified as *P. fluorescens* that were shown by both methods to have similar genotype. The same was true for two other *P. fluorescens* strains from G-BR. In addition, the genotypic distinctness of 19 antagonists identified as *P. putida* (eight isolates), *P. fluorescens* (eight isolates), *P. veronii* (one isolate), *P. taetrolens* (one isolate) and *P. alcaliphila* (one isolate) was revealed by both methods. However, a large number of the *Pseudomonas* antagonists that shared similar *gacA* types corresponded to different BOX-PCR groups. This incongruence may stem from the differences in the level of resolution of both methods: while BOX-PCR targets random site in the genome and thus provides information on the genomic diversity, gacA-DGGE analysis is based on one specific gene. Therefore it would be expected that strains having similar *gacA* types can posesse different genomic profile. In addition, similar DNA sequences differing at maximum of four to six nucleotides could possess the same electrophorectic mobility on the DGGE gels (Frapolli et al., submitted), this may also be the case for some of the antagonists which shared similar *gacA* types but possessed different BOX-PCR types. Cultivation-independent analysis of *gacA* gene in the bulk soils, from where

the antagonists were isolated, revealed a high diversity and site-specific *Pseudomonas gacA* gene assemblage in each soil. Pseudomonads population in these soils were better distinguished by DGGE analysis of amplified *Pseudomonas*-specific *gacA* gene fragments (this study) than by *Pseudomonas*-specific 16S rRNA gene fragments (Lembke, in preparation). This was evidence when better resolution and more DGGE bands indicating a higher diversity of *Pseudomonas* population were found with the latter than the former. These results corroborates the results of Costa et al. (2007), where a higher resolving potential was reported in the DGGE profile of bulk and rhizosphere soils with analysis of gacA-based *Pseudomonas* than 16S rRNA-based *Pseudomonas.*

When *gacA* types derived from the culturable *Pseudomonas* antagonists were linked with the culture-independent *gacA*-DGGE profiles from community DNA of each soil, only few culture-derived *gacA* types were represented in their corresponding *gacA* community patterns. Possible explanation for many of the unmatched *gacA* DGGE bands is that our collection only contained a small fraction of the culturable *Pseudomonas* spp*.* with antagonistic activity toward *R. solani* and *F. oxysporum*. Obviously a culturable fraction of this genus that was not antagonistic to the test pathogens have been left out of our collection*.* Also, *s*ome *Pseudomonas* species which are less abundant and would be rather difficult to recover by cultural isolation due to competition with other numerous *Pseudomonas* species were also represented in the *gacA* community.

Chapter 5: Monitoring rhizosphere competence, biological control and the effects on the soil microbial communities of *in vitro* antagonists towards *R. solani* tested on lettuce plants.

Even though *in vitro* media-based isolation of antagonistic microorganism has been used by several authors to retrieve successful biocontrol agents, some drawbacks are still associated with the method. The main drawback of the method is lack of correlation between *in vitro* antagonistic activity and field performance for some microbial antagonists (Schottel et al., 2001; Faltin et al., 2004). Therefore, to guarantee the efficacy of an *in vitro* antagonist, it must be able to colonize and survive in the rhizosphere of the host, and efficiently protect the host plant against the harmful effects of the pathogen in an environment, which simulates field conditions. Having this in mind, in Chapter 5 of this thesis growth chamber experiments carried out with lettuce as the chosen model plant are reported. Ten antagonists were selected based on *in vitro* inhibition towards *F.oxysporum* and/or *R. solani* AG3 and their root colonization efficiency in a pre-screening greenhouse experiment. Similarly, all displayed in *vitro* activity towards *R. solani* AG1-IB, the pathogen of the model plant (lettuce). The experiment aimed at investigating the potential of the ten *in vitro* antagonists in controlling bottom rot disease caused by *R. solani* AG1-IB on lettuce plants in growth chamber experiments, and to evaluate the antagonists for their survival and root colonization efficiency by means of selective plating. Also the influence of the best antagonists on microbial communities in the rhizosphere of lettuce was assessed using cultivation-independent method (PCR-DGGE analysis). Evaluation of the root colonization of the antagonists by selective plating showed that most inoculant strains could be detected at approximately 5×10^6 g^{-1} fresh root weight after three weeks of sowing. Nonetheless, most antagonists did not suppress the disease caused by the pathogen. In the first growth chamber experiment involving the ten antagonists, only four antagonists (three identified as *Pseudomonas fluorescens* and one as *Pseudomonas jessenii*) significantly decreased disease severity on lettuce plants. While in the subsequent three

experiments with the four best antagonists, only *P. jessenii* RU47 effectively and consistently suppressed the pathogen. In line with reports from several other authors (Schottel et al., 2001; Faltin et al., 2004), in this study we found discrepancy between *in vitro* antagonists activity and *in vivo* pathogen suppression, as six of the selected antagonists that displayed *in vitro* activity against *R. solani* AG1-IB failed completely to suppress the pathogen in the growth chamber experiments. Also for three of the best four antagonists under growth chamber conditions, we observed inconsistency in their biological control activity. Inconsistent performance of biological control agents in disease suppression has been identified as one of the major concerns in the use of biocontrol agents (Faltin et al., 2004; Haas and Defago, 2005). Inefficient root colonization, loss of ecological competence and insufficient or late production of antifungal metabolites, among others, are the factors assumed to be responsible for this inconsistency (Bloemberg and Lugtenberg, 2001; Haas and Defago, 2005; Mark et al., 2006). However, we observed almost similar level of cell populations for most antagonists after three weeks of inoculation; this implies that inability of these isolates to suppress disease was not due to insufficient root colonization. The inability of the isolates to exert their antagonistic activity in the rhizosphere may be due to colonization of the *in vitro* antagonists at locations different from the infectious site of the pathogen or insufficient cell density of the antagonists at the infectious site. Since colonization patterns were not investigated, there is no evidence to indicate differences in colonization sites of the antagonists and the pathogen.

Despite the potential benefits of BCA in plant protection, their introduction into the environment is often associated with public concern, particularly relating to their influence on microbial community structure (displacement and pathogenicity effect on the non-target organisms) and processes that are essential to general soil ecosystem functioning (Winding et al., 2004). Effects of BCAs on fungal and bacterial community

structure have been repeatedly found (Winding et al., 2004; Götz et al., 2006) while for some study no effect was observed (Lottmann et al., 2000; Viebahn et al. 2003). Thus, a necessary condition for introducing a biocontrol agent into the environment is that the effects of the introduced BCA on non-target organisms should be at least tolerable, if not negligible (Winding et al., 2004). In this respect, the survival of *P. jessenii* strain RU47 and its effect on the relative abundance of dominant bacterial, fungal and *Pseudomonas* communities in the rhizosphere of lettuce plants was evaluated by means of DGGE analysis of 16S/18S rRNA or *gacA* gene fragments amplified from total community DNA. We found that RU47 belonged to the dominant ribotypes of *Pseudomonas* and *gacA*-containing *Pseudomonas* community as dominant band corresponding to electrophoretic mobility of RU47 was detected only in the plants treated with RU47. However, in the bacterial community an unequivocal detection of RU47 was impossible due to the complexity of the DGGE pattern. The inoculation of RU47 had rather no effects on the *Pseudomonas* whether 16S rRNA- or *gacA*-gene based community throughout the seven-week growing period. Effects of the inoculation with RU47 on the fungal communities were observed as few ribotypes were detected in the DGGE patterns of the control plants which were missing in the RU47 treated plants. Since activities of BCAs are not directed to a unique or specific pathogen, non-target effects can be expected. We also found evidence that RU47 inhibited the growth of *R. solani* AG1-IB as indicated by a considerable decrease in the relative abundance of the pathogen in the rhizosphere of lettuce inoculated with RU47. Overall, our results revealed that *P. jessenii* RU47 was the only strain out of the ten selected *in vitro* antagonists, which could consistently suppress bottom rot disease caused by *R. solani* on lettuce plants. Interestingly, for RU47 we found no relationship between levels of *in vitro* inhibition and biological control, as the strain only had a weak inhibitory activity against *R.*

solani AG1-1B *in vitro*. The mechanisms by which this isolate suppresses *R. solani* AG1-IB in lettuce is not known. However, *in vitro* assay revealed only protease and siderophores synthesis in strain RU47. In addition, HPLC analysis of the culture filtrates of RU47 detected no known anti-fungal metabolites except a small-size peptide. Thus the exact mechanism of activity of RU47 remains a subject for further investigation.

In conclusion, isolation of bacterial antagonists towards *R. solani* AG3 and *F. oxysporum* in the six different soils that originated from five ecological zones in Europe, gave us an overview of the proportion, composition and diversity of bacterial antagonists in each soil. Overall our data indicated that each soil harbored different proportions and compositions of bacterial antagonists of *R. solani* AG3 and *F. oxysporum*. The diversity was indeed higher for suppressive soil, particularly the French soil (FR), than for soil without history of suppression. The consortium of bacteria with different biocontrol properties antagonizing the same pathogen found in each soil, especially the suppressive soils, suggested the reason for the effective and consistent disease suppression often observed in suppressive soils. While bacterial genera (*Streptomyces, Pseudomonas and Bacillus*) which have received long-time attention in biocontrol studies were frequently found in nearly all soils; bacterial genera such as *Dyella*, *Ochrobactrum*, *Brevibacillus* and *Variovorax, a*mong others, that have gained less attention and which could be exploited for future study on biological control of plant pathogens were revealed. Siderophore and protease production were prominent traits among the antagonists. We found evidence that siderophore production may be one of the mechanisms used by *Pseudomonas* spp. to antagonize the bacterial pathogen (*R. solanacearum*). The probably role of *Pseudomonas* antagonists carrying 2,4-DAPG encoding gene in the suppressiveness of Swedish soil was revealed, as this group belong to the dominant antagonistic

bacterial group isolated from this soil. Despite the failure or inconsistency of the majority of the selected antagonists in controlling *R. solani* AG1-IB in growth chamber experiments, the success, efficacy and consistency of one strain, *P. jessenii*, is worth notice. However, the mechanism of activity of this strain could not be ascertained in this study and thus needs further investigation. The efficacy of *P. jessenii* to control bottom rot disease of lettuce caused by *R. solani* under growth chamber conditions is the basis for a research grant proposal submitted to the DFG in which we intend to explore the effect of the soil type. It is also an indication, that there could be some other potential biocontrol agents in our collection of antagonists.

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List of abbreviations

2,4-DAPG	2,4-diacetylphloroglucinol
AGS	Argine glucose salt
ANOVA	Analysis of variance
BCAs	Biocontrol agents
BSA	Bovine serum albumin
CANOCO	Software for canonical community ordination
CAS	Chrome azurol S
cfu	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleotides. 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxythymidine 5'-triphosphate (dTTP).
FAME	Fatty acid methyl ester
FR	France
g	gravity
gacA	Global antibiotics and cyanide control
G-BR	Germany (Braunschweig)
G-BS	Germany (Berlin)
H`	Shannon-Weaver index of diversity
HPLC	High performance liquid chromatography
ISR	Induce systemic resistance
KMB+	King´s B Medium supplemented with chloramphenicol and ampicillin

LB	Luria-Bertani
ng	nanogram
NL	The Netherlands
PCA	Phenazine-1-carboxylic acid; Principal component analysis
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
phlD	Gene within the 2,4-diacetylphloroglucinol biosynthetic loci involved in the synthesis of monoacetylphloroglucinol (MAPG).
Phz	Phenazine
phzCD	gene involved in biosynthesis of phenazine
pltC	gene involved in biosynthesis of pyoluteorin
PLT	Pyoluteorin
prnD	gene involved in biosynthesis of pyrrolnitrin
PRN	pyrrolnitrin
rpm	revolutions per minute
rRNA	Ribosomal ribonucleic acid
SE	Sweden
Taq	Thermus aquaticus
TSB	Trypticase soy broth
TZC	Triphenyl tetrazolium chloride
UK	The United Kingdom
UPGMA	Unweighted pair-group method using arithmetic averages
WA	Waksman agar
WAS	weeks after sowing
YPG	Yeast peptone glucose

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PUBLICATIONS

Adesina, M.F., Lembke, A., Costa, R., Speksnijder, A., Smalla, K. (2007) Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. Soil Biology and Biochemistry 39: 2818-2828.

Adesina, M.F., Lembke, A., Vatchev, T.D., Grosch R., Smalla, K. Monitoring rhizosphere competence, biological control and the effects on the soil microbial communities of *in vitro* antagonists towards *R. solani* tested on lettuce plants (manuscript to be submitted to Environmental Microbiology Journal).

Adesina, M.F., Costa, R., Lembke, A., Smalla, K. Diversity of *Pseudomonas* specific-*gacA* gene among culturable antagonistic *Pseudomonas* isolates and in the bulk soils using PCR-DGGE analysis (manuscript in preparation)

Appendix

Appendix 1. Compositional diversity at the species level of antagonists isolated from the six soils

OTU (specie level)	FR	NL	SE	UK	G-BR	G-BS
Alphaproteobacteria						
Bosea thiooxidans	1					
Ochrobactrum sp.	4					
Rhizobium leguminosarum	1					
Rhizobium sp.	1					
Sinorhizobium sp.	1					
Xanthobacter agilis	1					
Betaproteobacteria						
Achromobacter xylosoxidans	1					
Alcaligenes faecalis	1					
Burkholderia cepacia		1				
Burkholderia pickettii	1					
Collimonas fungivorans		1				
Delftia acidovorans				1		
Variovorax paradoxus	1		1	3		
Gammaproteobacteria						
Dyella japonica		1				
Dyemonas todaii		3				
Enterobacter ludwigii	1					
Enterobacter cancerogenus	1					
Lysobacter antibioticus	1					
Pantoea agglomerans	1					
Pseudomonas fluorescens	4	16	25	4	2	7
Pseudomonas putida	4	13	5		1	
Pseudomonas alcaliphila			1			
Pseudomonas cannabina		1				
Pseudomonas chlororaphis						1
Pseudomonas grimontii						1
Pseudomonas jessenii			5			4
Pseudomonas lutea			1			
Pseudomonas moraviensis		1				
Pseudomonas agarici		1	1			
Pseudomonas fulgida			1			
Pseudomonas mucidolens			1			
Pseudomonas veronii	1					
Pseudomonas taetrolens		1				
Pseudomonas syringae glycine			2			
Pseudomonas marginalis						1
Pseudomonas sp.	1		1	1		2
Stenotrohomonas maltophilia	12		4	2		3
Yersinia pseudotuberculosis	1					
Flavobacteria						
chryseobacterium balustinum				1		
Bacilli						
Bacillus vallismortis					2	
Bacillus subtilis					7	1
Bacillus cereus					1	1
Bacillus licheniformis						2
Bacillus sphearicus		1				
Bacillus pumilus	3					1
Bacillus mycoides		5				
Brevibacillus brevis					1	

OTU (specie level)	FR	NL	SE	UK	G-BR	G-BS
Brevibacillus parabrevis	1					
Paenibacillus polymyxa				1	2	
Paenibacillus kribbensis					1	
Staphylococcus schleiferi	1					
Kurthia-sibirica	1					
Actinobacteria						
Arthrobacter sp.	1			2		
Arthrobacter globiformis		2				
Arthrobacter-oxydas				2		
Brevibacterium antarcticum			1			
Kytococcus sedentarius		1				
Microbacterium flavescens	4					
Micrococcus sp.			1			
Micrococcus luteus	1			1		
Nocardiopsis dassonvillei			1			
Rhodococcus erythropolis			1			
Streptomyces caviscabies	2	1	11	4	3	2
Streptomyces achromogenes						1
Streptomyces ambofaciens	1					
Streptomyces ciscaucasicus					1	
Streptomyces exfoliatus						1
Streptomyces globosus	1					1
Streptomyces griseochromogenes			1			
Streptomyces griseus	4	1	2			2
Streptomyces indonesiensis					1	
Streptomyces kasugaensis				1		
Streptomyces macrosporeus						1
Streptomyces maritimus	3					
Streptomyces melanosporofaciens				1		
Streptomyces olivoreticuli					1	2
Streptomyces piloviolofuscus					1	
Streptomyces pulveraceus		9				1
Streptomyces purpeofuscus						1
Streptomyces purpureus		7				
Streptomyces resistomycificus	1					
Streptomyces roseoflavus	1					
Streptomyces ryensis						1
Streptomyces sampsonii						1
Streptomyces scabiei						1
Streptomyces sclerotialus		2				
Streptomyces setonii					1	
Streptomyces sparsogenes					1	
Streptomyces sporoclivatus		12			1	
Streptomyces thermotolerans	1					
Streptomyces tricolor					1	2
Streptomyces turgidiscabies					1	2
Streptomyces tumescens					1	
Streptomyces viridobrunneus	1				1	
Streptomyces sp.	1				1	
Streptomyces mutomycini	2					
Streptomyces olivochromogenes				1		
Unidentified						
Streptomyces-like	1	1	3	1	2	1
Others		1	1		1	